INNATE IMMUNE EVASION OF MICROBES

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ACADEMIC DISSERTATION

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To Linus, Marcus, and Marco

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PUBLICATIONS

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II. Meri, T., Amdahl, H.*, Lehtinen, M.J.*, Hyvärinen, S., McDowell, J.V., Bhattacharjee, A., Meri, S., Marconi, R., Goldman, A., Jokiranta, T.S. 2013. Microbes bind complement inhibitor factor H via a common site. *Plos Pathogens* 9 (4): e1003308

III. Amdahl, H. Jongerius, I., Meri, T., Pasanen, T., Hyvärinen, S., Haapasalo, K., van Strijp, J.A., Rooijakkers, S.H., Jokiranta, T.S. 2013. Staphylococcal Ecb protein and host complement regulator factor H enhance functions of each other in bacterial immune evasion. *Journal of Immunology*, 191(4):1775-84

IV. Amdahl, H, Tan, L., Haapasalo, K., Meri, T., van Strijp, J.A., Rooijakkers, S.H., Jokiranta, T.S. 2014. Staphylococcal protein Ecb impairs complement receptor-1 mediated recognition and phagocytosis of opsonized bacteria. Submitted.

* Equal contributions

ABBREVIATIONS

AP	Alternative pathway
BSA	Bovine serum albumin
C4BP	C4b binding protein
CD	Cluster of differentiation
CP	Classical pathway
CR	Complement receptor
CRP	C-reactive protein
DAF	Decay accelerating factor
Ecb	Extracellular complement binding protein
Efb	Extracellular fibrinogen binding protein
Fab	Fragment antigen-binding
FB	Factor B
Fc	Fragment crystallizible region
FD	Factor D
FcγR	Fc gamma receptor
FH	Factor H
FHA	Filamentous hemagglutinin
FHL-1	Factor H-like protein 1
FHR	Factor H-related protein
fMLP	Formylated peptides (N-formyl-Met-Leu-Phe)
FPR	Formyl peptide receptor
GPCR	G-protein coupled receptor
HIS	Heat inactivated serum
HSA	Human serum albumin
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LP	Lectin pathway
MBL	Mannose binding lectin
MCP	Membrane cofactor protein
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophil extracellular traps
NHS	Normal human serum
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PRR	Pattern recognition receptor
Ptx	Pertussis toxin
ROS	Reactive oxygen species
SCIN	Staphylococcal complement inhibitor
SCR	Short consensus repeat
SpA	Staphylococcal protein A
SSL	Staphylococcal superantigen-like
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor alpha
TP	Terminal pathway
VBS	Veronal buffered saline

ABSTRACT

Innate immunity is the first line of defense against microbes encountering the human body. A central player in initiating and augmenting the innate immunity is the complement system. Complement is a proteolytic and self-amplifying cascade consisting of soluble plasma proteins and membrane bound regulatory proteins. This system is able to recognize and discriminate between host and non-host structures via activation of three pathways: classical, lectin, and alternative pathway. All the pathways lead to formation of C3 activating enzymes resulting in covalent C3b deposition onto target cell surfaces. Deposition of C3b results in amplification of the activation, release of chemotactic molecules, attraction of phagocytes, and initiation of the terminal pathway resulting in formation of lytic membrane attack complexes.

To avoid destruction of host cells complement has to be strictly regulated. Factor H (FH) is the major regulator of the alternative pathway. It consists of twenty domains where the domains 1-4 bind to C3b and mediate inhibition of the complement activation while domains 19-20 are essential for the discrimination between host from non-host surfaces. FH downregulates complement activation by three means: acting as a cofactor in proteolytic inactivation of C3b, preventing formation of the C3 convertase, and accelerating decay of the C3 convertase.

Several pathogens utilize host FH to protect themselves against complement attack. Binding of FH provides the microbe with a powerful tool to evade, not only C3b-mediated opsonophagocytosis, but also inflammation, chemotaxis, and lytic membrane attack complexes. Several microbes have previously been shown to acquire surface-bound FH via its domains 5-7 and 19-20. While the number of pathogens reported to bind FH is increasing, it is known that some pathogens such as *Staphylococcus aureus* do not bind FH but instead bind C3b.

In this thesis there are three specific aims. First, to determine whether the important human pathogen, *Bordetella pertussis,* evades complement attack by the alternative pathway. Second, to discover by which mechanisms microbes bind FH via the C-terminal domains FH19-20. Third, to study how *S. aureus* prevents the attack by the alternative pathway of complement although it does not bind FH.

To study the first aim, various immunological assays were used to analyze binding of FH by different *Bordetella* strains. The results showed that both *B. pertussis* and the closely related *B. parapertussis* bound FH. The bound FH retained its regulatory activity for

factor I mediated cleavage of C3b, suggesting that the binding was important for serum survival.

To meet the second aim, i.e. to explain why the FH domains 19-20 is a common target for most FH-binding microbes, we used several FH19-20 point mutant proteins to analyze the binding sites on pathogens representing Gram-positive and Gram-negative bacteria, a yeast, and three purified microbial proteins. The results revealed a conserved binding site on the domain 20 for all the studied microbes. An explanation for the common binding site was provided by the fact that by binding to the domain 20, the microbial proteins enhanced the interaction between C3b and FH. This resulted in a more efficient inactivation of C3b and subsequently enhanced evasion of complement mediated damage.

The third aim of this thesis was to determine how pathogenic *S. aureus* evades the alternative pathway activation independently of direct FH-binding. For this we studied small secreted C3b-binding molecules of *S. aureus*, e.g. extracellular complement binding protein (Ecb) that binds to the same region as FH on C3b. We showed that FH deposition on the surface of *S. aureus* could be induced by the formation of tripartite complexes between Ecb, C3b, and FH. In the complex two of the regulatory functions of FH were maintained – and, most interestingly, even enhanced. The significance of the tripartite complex formation in complement evasion was verified using a serum sensitive *Haemophilus influenzae* strain. Ecb added to the serum before *H. influenzae* cells were included protected the bacteria from complement-mediated lysis, and the effect was increased by addition of the FH19-20. These results suggest that upon formation of tripartite complexes *S. aureus* may use FH for its own protection to eliminate C3b.

Studies with *S. aureus* revealed that the formation of the tripartite complex (Ecb:C3b:FH) did not enhance inactivation of the major opsonin C3b. This was surprising, since C3b is the ligand for complement receptor-1 (CR1) expressed on phagocytes. Therefore, we studied the role of the secreted proteins in inhibiting binding of C3b to neutrophils. The results revealed that Ecb blocks the interaction between C3b and CR1, which impaired the phagocytosis of C3b-opsonized *S. aureus* and presence of FH enhanced this. *S. aureus* is able to utilize host FH in three ways – to promote the tripartite complex formation so that the C3 convertase cannot be formed, to prevent CR1-binding leading to impaired opsonophagocytosis, and to prevent degradation of C3b to iC3b thereby preventing recognition of *S. aureus* by complement receptors 3 and 4 on phagocytes.

In conclusion, these studies have revealed an immune evasion mechanism for *B. pertussis* and identified in molecular detail a site on the domain 20 of FH important for various microbes in complement escape. These studies also explained how *S. aureus* uses soluble C3b-binding molecules to recruit host FH for immune evasion, despite not binding directly to this regulator. Taken together, these results revealed detailed knowledge on structural and functional basis that is relevant for vaccine and antimicrobial development.

1 INTRODUCTION

The environment is dominated by bacteria and every day our body has to defend itself against microbes. Our immune system, however, is very effective and only a minority of these microbes is able to enter and survive within the body. The innate immune system represents the first line defense against microbes and it can quickly prevent, control, or eliminate the incoming pathogen. It can also stimulate the second line of defense, the adaptive immune system, which reacts more slowly but is highly specific and has the ability to remember the same microbe if encountered in the body again.

Skin and mucosal surfaces provide effective protection against pathogenic microbes by inhibiting the entry of microbes to tissues and blood. If a pathogen succeeds in breaking through the host's physical barriers, molecules of the innate immunity are activated, such as antimicrobial agents and the complement system. The complement system targets foreign surfaces and mediates direct lysis and uptake by phagocytic cells.

The history of complement (originally called "alexin" by Buchner) began in the late 1880's when several researchers observed bactericidal activity in normal serum (Lachmann 2006). A Belgian microbiologist, Jules Bordet, performed the experiment that finally provided an explanation for this phenomenon. He demonstrated that a heat-labile blood component (complement) together with a heat-stable component (antibody) had the capacity to lyse certain bacteria.

For more than fifty years it was believed that complement required antibodies to be activated until Louis Pillemer demonstrated that complement can be activated independently from antibodies (Pillemer et al. 1954). He originally named the pathway "properdin pathway", now known as the alternative pathway (AP). The AP together with the lectin pathway (LP) appear to have been functional long time before the adaptive immunity (Farries & Atkinson 1991; Pinto et al. 2007). During the last decades several important discoveries within the complement field has broadened the research and it is no longer considered to be a non-specific immune response, but capable of discriminating between self and nonself. The importance of complement in immunity is emphasized by deficiencies or mutations of complement components.

2 REVIEW OF LITERATURE

2.1 THE IMMUNE SYSTEM

A well-organized network of cells and molecules comprises the immune system that responds to microbes or other foreign substances entering the human body. Its purpose is to recognize, immobilize and eliminate invading microbes and develop an immunological memory against previous pathogens. The two branches of the immune system, the innate and adaptive immunity, include a humoral and a cell-mediated part (Fig. 1). Although they are different in their mechanism of action, synergy between the parts is essential for a fully effective immune response.

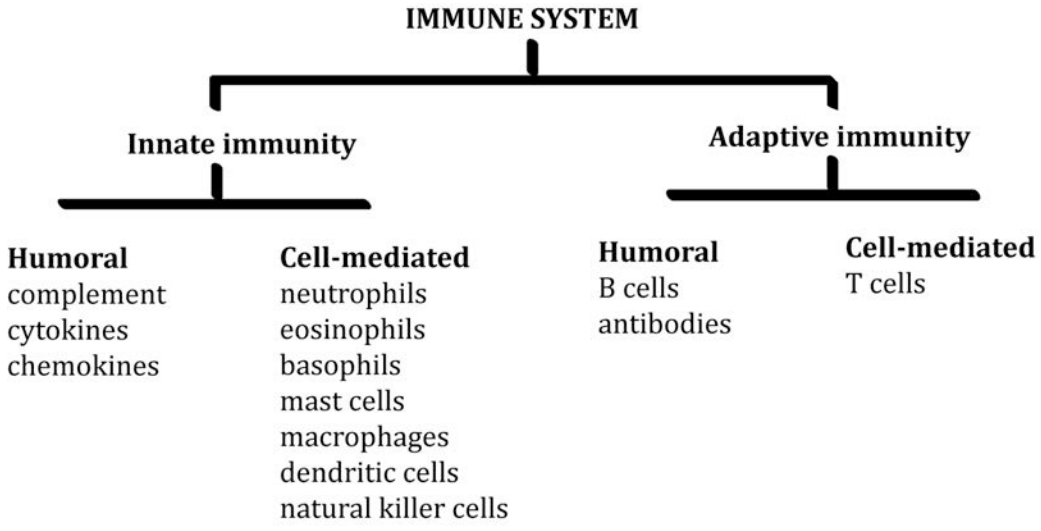

Figure 1. Schematic overview of the immune system.

2.1.1 INNATE IMMUNITY

Innate immunity is the evolutionarily oldest branch of host protection against invading pathogens. Tears and saliva contain lysozyme, defensins, and cathelicidins with antimicrobial activity that protect and hamper the growth of pathogens (Hancock & Diamond 2000; Ganz 2003). In addition, our microbiota present on the skin and on mucous membranes help to protect against pathogens by occupying sites that might be colonized by pathogens. If microbes manage to cross those barriers they encounter a

powerful and complex defense system consisting of proteins of the complement system (section 2.2) and phagocytic cells, including neutrophils (section 2.3).

The key to proper function of the immune system is its ability to discriminate self from nonself. Microbes can be recognized by pattern-recognition receptors (PRRs) present on host cell surfaces, endosomal vesicles, and even in the cytoplasm. The PRRs have signaling or phagocytic functions. Examples of phagocytic PRRs are the C-type lectin receptors, scavenger receptors (Peiser et al. 2002), and complement receptors (section 2.3.3). The G-protein coupled receptors (GPCRs) are signal receptors and they include the N-formyl peptide receptors (FPRs) (Rabiet et al. 2007) (section 2.3.2). The evolutionary conserved Toll-like receptors (TLRs) recognize commonly expressed molecules on microbes but not the host's own cells, and they are found both on cell surfaces and on endosomal vesicles (section 2.3.2). The Nod-like receptors (NLRs) are located in the cytoplasm where they detect intracellular microbes (Franchi et al. 2009).

There are three classes of phagocytic cells: monocytes and macrophages, dendritic cells, and granulocytes which include neutrophils, basophils, and eosinophils. Monocytes differentiate into macrophages upon entering various tissues where they can divide and are important in fast recognition and phagocytosis as well as in a later phase of the infection. The granulocytes are circulating in the bloodstream and migrate into tissues in response to an infection. Of these, neutrophils mediate the fastest response and can migrate in large numbers within a few hours to the microbial entry site (Kim et al. 2008). Macrophages and granulocytes are central in innate immunity since they can recognize and eliminate an invader without the help from the adaptive immunity. In contrast, the functions of the dendritic cells are primarily to process and present antigens to T cells, thereby linking innate and adaptive immunity (Nussenzweig et al. 1980; Steinman 2012).

Natural killer (NK) cells are found in blood and various tissues where they sense infected or abnormal cells without specific sensitization and eliminate them via apoptosis. Moreover, natural killer cells secrete cytokines that activate macrophages (Vivier et al. 2008).

Phagocytes are attracted to the site of infection by molecules of microbial or host derived origin such as complement fragments, chemokines and cytokines. Also, mast cells and e.g. histamine are involved in this process. These chemoattractants activate phagocytes and assist their migration through the blood vessel wall (Campbell et al. 1998).

Cytokines are proteins secreted by phagocytes and are essential in regulating and mediating cellular effector functions. Tumor necrosis factor alpha (TNF-α) activates endothelial cells to express selectins for recruitment of phagocytes to the infection site. Host defense against certain pathogens such as mycobacteria and some viruses is significantly impaired if TNF-α or its receptor are lacking (Havell 1989; Mohan et al. 2004). Mononuclear phagocytes are stimulated by TNF-α to secrete interleukin (IL)-1 that has similar functions as TNF-α. In local infections, low concentrations of TNF-α are generated, however, in severe Gram-negative sepsis (septic shock) TNF-α together with other cytokines, are produced in large amounts in response to lipopolysaccharide (LPS). This cytokine storm affects the complement and coagulation cascades, leading to endothelial damage and multiple-organ system failure.

Chemokines are small polypeptides that stimulate and regulate the migration of phagocytes and other leukocytes from blood vessel to inflamed tissues. Chemokine receptors are GPCRs found mainly on T cells.

2.1.2 ADAPTIVE IMMUNITY

The evolutionally more recent adaptive immune system provides a more versatile although a slower protection against microbial infections. The cells in innate immunity are, however, needed for initiation of the adaptive immune response. Antigen presenting cells, such as dendritic cells, take up pathogens and migrate to the local lymph nodes where they present the processed antigen to resting T cells. This results in activation and proliferation into T helper cells (CD4+) and cytotoxic T cells (CD8+). At the site of infection, T helper cells assist in activation of macrophages for more efficient phagocytosis whereas cytotoxic T cells directly kill infected cells or cells that lack protective receptors. A portion of the helper T cells assists antigen activated B cells to differentiate into antibody-producing plasma cells. Although the response to the antigen is slow, a long lasting memory to the specific antigens is developed.

Antibodies are soluble glycoproteins produced by B cells in response to antigens. Recognition and binding of antibodies to antigens can lead to neutralization of a virus, toxin, or labeling (opsonization) of the target for attack by other components of the immune system.

Antibodies are comprised of two identical heavy chains and two identical light chains and both heavy and light chains have a constant and variable region (Fig. 2). Based on the constant domain they are divided into five types: Immunoglobulin (Ig) A, IgD, IgE,

IgG, and IgM. IgG and IgM have important functions in complement activation and IgG has also a direct role in opsonization. IgA is produced mainly by mucosal lymphoid tissue, and subsequently forms an important part of defense against microbes entering the respiratory or gastrointestinal tract. IgD has a function in defense against respiratory pathogens by directly binding to pathogens or their virulence factors. It also induces signals that activate the production of antimicrobial molecules by several cells (Chen et al. 2009). IgE has a function in defense against helminths.

The heavy and light chains form two variable antigen binding fragments (Fab). The heavy chains have also a constant crystallizable domain (Fc) that is connected to Fabs via a flexible hinge region. Each Fab fragment recognizes and binds its target with high affinity, whereas the Fc region interacts with Fcγ-receptors (FcγRs) on leukocytes.

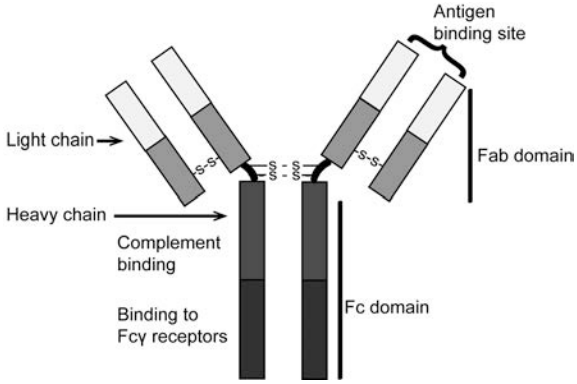

Figure 2. Structure of an IgG antibody. The Y-shaped antibody consists of two heavy and two light chains linked together by disulphide bonds (s-s) as indicated. The antigen binds to the variable region of the Fab domain while Fc region interacts with the receptor on the cell and mediates complement activation.

Human IgGs comprise four structurally different subclasses, IgG1-4, that differ in their heavy chain (γ-chain). The glycosylation of the immunoglobulins helps to stabilize the Fc region (Mimura et al. 2001) and glycans on the Fc region of IgG are even required for optimal interaction to its specific FcγR and subsequently the effector functions (Jefferis & Lund 2002). Immunoglobulins can also bind directly to glycans on pathogens and mannose receptors on phagocytes (Malhotra et al. 1995; Dong et al. 1999). The capacity to activate complement is highest for IgG3 followed by IgG1 and IgG2, whereas IgG4 completely lacks this ability.

2.2 THE COMPLEMENT SYSTEM

An organized network of more than thirty soluble or membrane-associated proteins comprises the powerful complement system. Based on its recognition capacity and its effector mechanisms, leading to either activation or downregulation of the cascade, affect both innate and adaptive immunity. By marking pathogens with opsonizing molecules and generating chemotactic molecules, complement attracts phagocytes and enhances phagocytosis of the invader. Receptors on erythrocytes bind C3b-opsonized immune complexes, debris from the body for transportation and clearance in the liver or spleen. Furthermore, the cleavage products of the complement cascade are recognized by B cells leading to downstream activation, antibody production and amplification of the immune response against those structures that have activated complement (Fearon & Locksley 1996; Carroll 2004; Kemper & Atkinson 2007; Pekkarinen et al. 2013).

2.2.1 RECOGNITION

Effector functions of complement depend on target versus host discrimination and each of the three complement pathways uses its own unique recognition mechanism. Based on the recognition, complement proteins act together and make sure that the complement cascade is regulated correctly by either promoting or halting the cascade.

Three strategies are used by the immune system to detect unwanted cells or particles for elimination: microbial nonself, missing self, and altered self (Medzhitov & Janeway 2002). Recognition of targets by the complement system can also be divided into these three categories.

2.2.1.1 MICROBIAL NONSELF

The host PRRs can detect unique conserved patterns on microbes referred to as pathogen-associated molecular patterns (PAMPs). These patterns are not found on host cells but on microbes and are usually vital microbial products like LPS, peptidoglycan, or flagella (Janeway 1989). Direct recognition of microbial nonself by complement is mediated via molecules such as C1q, mannose-binding lectin (MBL), and ficolins. C1q recognizes antigen-bound antibodies, MBL recognizes mannose and N-acetylglucosamine, whereas ficolins bind N-acetylglucosamine residues on microbial surfaces (Matsushita & Fujita 1992; Matsushita 2010).

2.2.1.2 MISSING SELF

Host cells possess membrane-bound complement proteins that downregulate the complement activity on own surfaces. Therefore, the absence of these regulators makes the surface much more prone to complement activation. Even in this case the fluid phase AP regulator factor H (FH) can protect self structures. Discrimination between self and nonself targets is mediated by binding of FH to sialic acids and glycosaminoglycans on self cells (Kazatchkine et al. 1979; Meri & Pangburn 1990; Pangburn 2002; Kajander et al. 2011). If the cell is missing molecules that mark them as self they will be destroyed by the immune system.

2.2.1.3 RECOGNITION OF ALTERED SELF

Removal of apoptotic and necrotic cells is crucial for the cell homeostasis and complement is suggested to have a role in the late apoptotic phase. The classical pathway component C1q, as well as C-reactive protein (CRP) (Jiang et al. 1991) and pentraxin 3 (PTX3) (Bottazzi et al. 1997; Deban et al. 2010) have been shown to bind apoptotic and necrotic cells leading to opsonization and phagocytosis of the target (Elward et al. 2005; Trouw et al. 2007). The recruitment and function of soluble regulators such as C4b-binding protein (C4BP) and FH, are most probable to inhibit excessive complement activation and avoid inflammation (Elward et al. 2005; Trouw et al. 2005; Trouw et al. 2007).

2.2.2 ACTIVATION

After recognition, the activation of complement occurs via the classical, lectin, or alternative pathway. All pathways converge into the C3 molecule and formation of the C5 convertase that initiates the terminal pathway.

2.2.2.1 CLASSICAL PATHWAY (CP)

The CP is activated when the C1q molecule of the C1 complex binds to its ligand (Table 1). The major ligands are the Fc-regions of IgG or IgM antibodies that have formed complexes with an antigen (Bindon et al. 1988; Collins et al. 2002a). At least two molecules of IgG bound in close proximity on the target are required for interaction with C1q (Hughes-Jones et al. 1984). However, C1q can also bind several targets in the absence of antibodies, like PTX3 (Bottazzi et al. 1997; Deban et al. 2010) CRP (Jiang et al. 1991), lipid A of LPS (Morrison & Kline 1977) and viral envelope glycoproteins (Cooper et al. 1976). C1q is a complex protein consisting of monomers with collagen-like stalks

and globular heads arranged in trimers. Six trimers form the C1q molecule which appears like a bunch of tulips in electron microscopy (Calcott & Muller-Eberhard 1972; Gadjeva et al. 2008). There is no enzymatic activity in C1q. C1q binding to its ligand results in conformational changes and activation of the proteases C1r and C1s (Naff & Ratnoff 1968; Dodds et al. 1978; Budayova-Spano et al. 2002). The C1s molecule in the activated C1 complex cleaves C4 and C2 in a Ca^{2+}-dependent manner (Lepow 1963; Ziccardi & Cooper 1977; Bally et al. 2009).

The C4 molecule consists of three chains (α, β, γ) that are bound together by disulfide bridges and non-covalent interactions. Upon activation, C1s cleaves C4 to yield C4b and C4a (Schreiber & Müller-Eberhard 1974) and a thioester group is exposed on C4b. This highly reactive group can attach covalently to a nearby surface while the C4a fragment acts as a weak chemoattractant (Gorski et al. 1979; Isenman & Kells 1982; Dodds et al. 1996). In the presence of Mg^{2+}, the membrane bound C4b binds the single chain C2 molecule allowing its cleavage by C1s resulting in formation of the C3 convertase C4b2a (Müller-Eberhard et al. 1967; Nagasawa & Stroud 1977). It is not exactly clear what role the smaller dissociated C2b fragment has. One study suggested that it enhances the vascular permeability leading to edema (Strang et al. 1988), however, the findings are not confirmed. Nevertheless, domains within C2b are important for binding of intact C2 to C4b (Nagasawa & Stroud 1977).

Table 1. Components of the classical pathway

Component	Molecular mass (kDa)	Function
C1	780	
C1q	460	Binds to Fc of IgG, IgM
C1r	80	Cleaves C1s
C1s	80	Cleaves C4 and C2
C4	205 (α, β, γ chains)	Binds to surfaces
C4a	9	Chemoattractant
C4b	198	Covalently bound to the surface
C2	102	Binds C4b
C2a	73	Cleaves C3
C2b	34	Unknown

2.2.2.2 LECTIN PATHWAY (LP)

The lectin pathway is activated when carbohydrate based ligands such as mannose or N-acetylglucosamine on microbes are recognized by MBL or ficolins (1-, 2-, or 3-ficolin) (Table 2). Interaction of these recognition molecules with the target leads to activation of the MBL-associated serine proteases (MASP-1, MASP-2, and MASP-3), which activate downstream complement molecules (Matsushita & Fujita 1992; Sato et al. 1994; Thiel et al. 1997). MASP-2 cleaves both C4 and C2 forming the C3 convertase, C4b2a, while MASP-1 can activate MASP-2 and is suggested to have a major role in the activation of the lectin pathway (Degn et al. 2012; Heja et al. 2012; Megyeri et al. 2013). Both MASP-1 and MASP-2 have autoactivating properties as they are found to associate in the same complex indicating that they act in a similar fashion as C1r and C1s do (Degn et al. 2012). The physiological role of MASP-3 is not yet clear but, however, murine MASP-3 and MASP-1 have shown to cleave the pro-factor D, indicating involvement in the AP activation (Takahashi et al. 2010; Sekine et al. 2013).

Table 2. Components of the lectin pathway

Component	Molecular mass (kDa)	Function
MBL	3-6 x 32 kDa	Binds mannose or N-acetylglucosamine
Ficolins 1,2, and 3	12-18 x 34-40 kDa	Bind acetylated molecules
MASP-1	100	Cleaves C2/activates MASP-2
MASP-2	76	Cleaves C4 and C2
MASP-3	94	Cleaves pro-factor D?

2.2.2.3 ALTERNATIVE PATHWAY (AP)

All three complement activation pathways converge at the stage of C3, the key molecule of complement activation. It has the highest concentration of the complement components in plasma (0.7-1.5 mg/ml). The 185 kDa molecule is composed of α (115 kDa) and β (75 kDa) chains which are held together by one disulphide bond and noncovalent forces (Tack et al. 1979a; Janssen et al. 2005) (Table 3).

Activation of AP in a fluid phase is spontaneous since C3 is hydrolyzed at a low rate to form C3(H_2O) (Pangburn et al. 1981) (Fig. 3). The metastable C3(H_2O) is highly reactive and within milliseconds it can bind factor B (FB) in the presence of Mg^{2+} making it susceptible to cleavage by factor D (FD) and generating the initial fluid phase convertase, C3(H_2O)Bb.

The initial fluid phase convertase, C3(H_2O)Bb, can cleave C3 into C3b and the chemoattractant C3a (Hugli 1975). The formed C3b can attach to basically any surface via its exposed thioester domain within a very short time (Fearon & Austen 1975a; Law & Levine 1977; Pangburn & Müller-Eberhard 1980; Sim & Sim 1981; Fishelson et al. 1984).

Table 3. Components of the alternative pathway

Component	Molecular mass (kDa)	Function
C3	185	Binds covalently to surfaces
C3a	9	Chemoattractant
C3b	175	Binds FB, FH, CR1
FB	93	Binds C3
Ba	30	
Bb	60	Cleaves C3 and C5
FD	26	Cleaves pro-factor D?
Properdin	2-5 x 53	Stabilizes C3/C5 convertase

A conformational change occurs when FB binds to C3(H_2O) or C3b and exposes a binding site for the serine protease FD (Lesavre & Müller-Eberhard 1978; Janssen et al. 2009; Hourcade & Mitchell 2011). This results in a release of the Ba fragment while leaving the active subunit Bb bound to the C3 convertase, C3bBb (Medicus et al. 1976b; Lesavre et al. 1979). Amplification of the AP occurs when C3 convertase cleaves and activates other C3 molecules to C3b resulting in opsonization of the target (Müller-Eberhard & Götze 1972). The C3 convertase has a half-life of only ninety seconds (Medicus et al. 1976a; Pangburn & Müller-Eberhard 1986) but can be stabilized by associating with properdin (Fearon & Austen 1975b). Even though C3b surface deposition is a result of activation of all pathways, most of the deposited C3b molecules are generated by the AP C3 convertase (Harboe & Mollnes 2008).

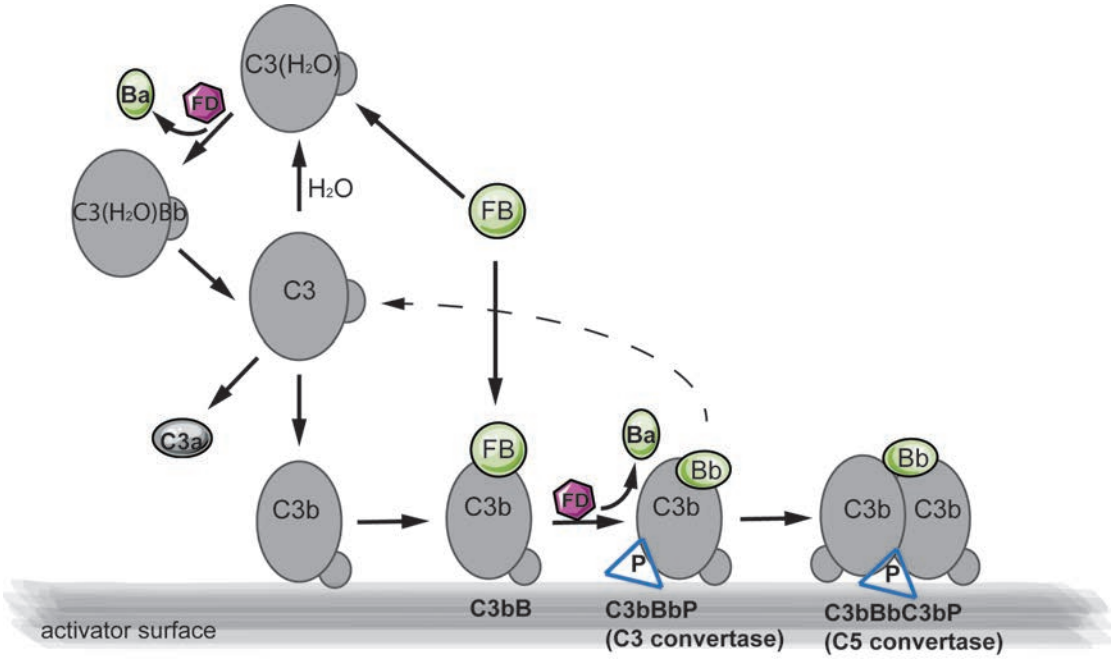

Figure 3. Activation of the AP. C3 is spontaneously hydrolyzed at a low rate in plasma forming C3(H_2O) which binds factor B (FB) and becomes cleaved by factor D (FD). The formed fluid phase convertase, C3(H_2O)Bb, cleaves C3 into C3b and C3a. On an activator surface, FB binds to the deposited C3b molecule making it susceptible to cleavage by FD resulting in the release of the Ba fragment and formation of the C3 convertase, C3bBb. A new C3b molecule binds to the surface deposited C3b and forms the C5 convertase, C3bBbC3b. Properdin (P) increases the half-lives of the C3 and C5 convertases when bound to the complexes. The C3 convertase cleaves other C3 molecules as indicated by the dotted arrow (amplification).

2.2.2.4 TERMINAL PATHWAY (TP)

The binding of an additional C3b on or near the C3 convertase generates the C5 convertase, C4b2aC3b (CP/LP) or C3bBbC3b (AP) (Daha et al. 1976; Medicus et al. 1976a; Rawal & Pangburn 2001; Pangburn & Rawal 2002) and initiates the TP (Fig. 4) and formation of the membrane attack complex (MAC) (Müller-Eberhard 1986). The C5 molecule binds to surface bound C3b in the C5 convertase and is cleaved by C2a or Bb into C5b and the chemoattractant C5a (Medicus et al. 1976b). C5a is rapidly metabolized by plasma and cell surface peptidases to form C5a-desArg (Bokisch & Müller-Eberhard 1970). C5a, and to a lesser extent C5a-desArg, are important chemotactic peptides in recruitment and activation of phagocytes to the site of infection or inflammation by binding to the C5a receptor (C5aR) on phagocytes. C5b remains attached to the C3b in the complex when the single chain molecule C6 binds to C5b (Tack et al. 1979b). A

binding site is exposed for the next molecule, C7 that results in a conformational change within the C5b-7 complex which is released to the fluid phase (Podack et al. 1978a). The formed complex is very unstable and is inactivated by hydrolysis or other plasma proteins, unless it binds to a membrane within short time (Podack et al. 1978b). When bound to a membrane, it attracts the three-chained C8 molecule, which uses its β-chain to bind to C7 resulting in the C5b-8 complex (Tamura et al. 1972). The last component in the pathway, the single-chained C9, binds to the C8α-chain and undergoes a conformational change exposing a binding site for additional C9 molecules. Numerous C9 molecules form a ring-like structure in the membrane, a pore, known as the MAC. The functional consequence of the MAC formation is osmotic lysis of the cell.

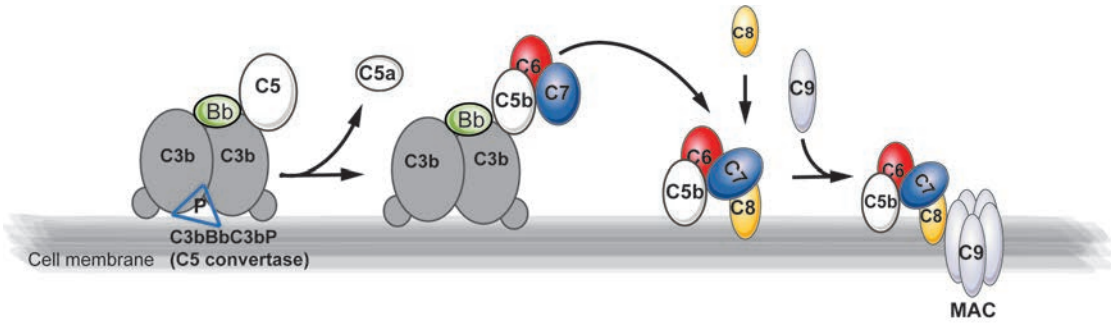

Figure 4. Activation of the terminal pathway. The C5 convertase cleaves bound C5 to C5a and C5b. C5b in a complex with C6 and C7 binds to the membrane and attracts C8 and C9 resulting in the formation of the membrane attack complex (MAC), followed by lysis of the cell.

2.2.3 REGULATION

Since complement is continuously activated at a slow rate and is amplified in plasma, uncontrolled activation would lead to local tissue damage and a rapid consumption of the complement components. Therefore, the cascade is strictly regulated at almost every step by several regulators in the fluid phase and on membranes. The importance of regulators is evident in individuals who are deficient in, or have altered regulators leading to various severe diseases. Microbes can also use the host regulators to protect themselves against complement attack (section 2.4). Most of the regulators are members of the regulators of complement activation (RCA) cluster located in chromosome 1q32. Regulators share a similar structure called short consensus repeat (SCR), complement control protein (CCP) domains, or Sushi repeats (Medof et al. 1987b). A single SCR is a globular domain containing 60-70 amino acids with a

hydrophobic core wrapped in β-sheet held together by two intradomain disulphide bonds (Klickstein et al. 1987). The RCA proteins contain multiple copies of these domains, from four (as for decay accelerating factor, DAF; and membrane cofactor protein, MCP) to thirty (complement receptor 1, CR1). Domains are organized in such a way that proteins have a typical elongated structure.

2.2.3.1 SOLUBLE REGULATORS

C1-inhibitor (C1INH) is a single chain highly glycosylated serine protease (Johnson et al. 1971; Gregorek et al. 1991) belonging to the serine protease inhibitor (serpin) family (Table 4). In addition of being able to inhibit the CP/LP, it is also capable of inhibiting the fibrinolytic and contact/kinin system of coagulation such as Factor XIa and XIIa, kallikrein and plasmin (Ratnoff & Naff 1969; Forbes et al. 1970; Gigli et al. 1980). C1INH has two important roles in regulation of C1. In the absence of antibody, the C1 complex will autoactivate at low levels and C1INH is needed to prevent this by stabilization of the complex and therefore preventing the consumption of C1, C2 and C4 (Ziccardi 1982; Hosoi et al. 1987; Bianchino et al. 1988). The second role of C1INH is to directly inactivate C1 by binding to the activated C1r and C1s which results in dissociation of those from C1q yielding one complex of one C1r, one C1s and two molecules of C1INH (Ziccardi & Cooper 1977; Perkins et al. 1990). A deficiency of C1INH results in a disease hereditary angioedema (HAE) which is characterized by episodic edema of the extremities, face, larynx or gastrointestinal tract.

Table 4. Soluble complement regulators.

Regulator	kDa	Serum conc. (μg/ml)	Function	Reference
C1INH	71	200-250	Inactivates C1r, C1s	(Gregorek et al. 1991)
C4BP	540-590	200	CA[1], DAA[2]	(Fujita et al. 1978; Fujita & Nussenzweig 1979; Dahlbäck & Hildebrand 1983)
FH	155	233-269	CA, DAA	(Pangburn et al. 1977; Ross et al. 1983)
FHL-1	42	10-50	CA, DAA	(Kühn et al. 1995)
Factor I	90	35	Cleaves C3b/C4b	(Harrison & Lachmann 1980)
Properdin	53	25	Stabilizes C3bBb	(Fearon & Austen 1975b)
Vitronectin	84	250-540	Inhibits MAC[3]	(Podack et al. 1984)
Clusterin	80	250-420	Inhibits MAC	(Murphy et al. 1989)

[1]cofactor activity; [2]decay accelerating activity; [3]membrane attack complex

C4b-binding protein (C4BP) regulates both CP and LP. By interfering with the C4b molecule within the C4b2a enzyme on cell surfaces, C4BP accelerates its natural decay and prevents an association of the convertase subunits (Gigli et al. 1979). Furthermore, it acts as a cofactor for factor I in proteolytic cleavage of C4b to the fragments C4c and C4d in both fluid phase and on membranes (Fujita & Nussenzweig 1979; Fujita & Tamura 1983). At high concentrations it can also accelerate the decay of the AP C3 convertase (Blom et al. 2003) but the physiological role of this is not verified *in vivo*.

C4BP is a large glycoprotein produced mainly in the liver. It has several isoforms, and the major form consists of seven α-chains (each 70 kDa) and a single β-chain (45 kDa) (Dahlbäck et al. 1983). α and β-chains are linked by disulphide bridges and organized in a spider-like structure where the chains are linked to a central "core" in the carboxyl-terminal ends (Dahlbäck et al. 1983; Chung et al. 1985; Hillarp & Dahlbäck 1990). Each α-chain consists of eight SCR domains where the amino-terminal domains 1 and 2 are required for binding to C4b and the cofactor activity (Chung et al. 1985; Blom et al. 2000). The β-chain has three SCR domains and it binds with protein S, most likely via the first two domains (Hillarp & Dahlbäck 1990; van de Poel et al. 1999). Protein S is a vitamin K-dependent protein involved in the coagulation cascade. Although the C4BP-protein S complex has been shown to have some anticoagulant activity its role is not fully understood.

Factor H (FH), originally named β1H-globulin, is the major fluid phase regulator of AP (Nilsson & Müller-Eberhard 1965). FH controls the AP at the C3 level by three mechanisms as illustrated in figure 5. It regulates the activation of C3 by acting as a cofactor for factor I in cleavage of C3b resulting in an inactive form, iC3b (Pangburn et al. 1977). *In vivo* it is observed that FH can assist the serine protease factor I in cleaving C3b at two sites while under non-physiological conditions *in vitro* further cleavage of C3b can be observed (Ross et al. 1983). In addition, FH competes with FB for binding to C3b and accelerates the natural decay of the AP C3 convertase, C3bBb (Weiler et al. 1976; Whaley & Ruddy 1976), by dissociating the Bb-fragment from the complex (Pangburn & Müller-Eberhard 1978).

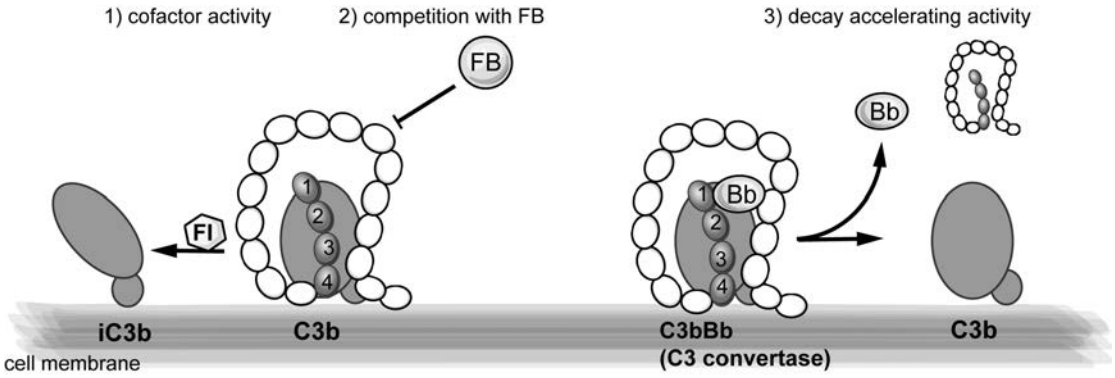

Figure 5. Regulation of the AP by factor H. 1) Cofactor activity: FH associates with C3b and promotes proteolytic cleavage of C3b by factor I (FI) resulting in an inactive form of C3b (iC3b). 2) FH competes with FB in binding to C3b. 3) Decay accelerating activity: FH associates with C3bBb and enhances the dissociation of the Bb from the complex.

FH is an elongated molecule consisting of twenty SCRs (Sim & DiScipio 1982; Ripoche et al. 1988) (Fig. 6). The regulatory site of FH to C3b that contains the cofactor activity and decay accelerating activities is located within the domains 1-4 (FH1-4) (Gordon et al. 1995; Kühn et al. 1995; Kühn & Zipfel 1996; Wu et al. 2009). The other important C3b binding site is within the C-terminal domains 19-20 (FH19-20), which is also the surface recognition site and has high affinity for anionic molecules like sialic acids and glycosaminoglycans abundantly found on host cells (Jokiranta et al. 2000; Pangburn 2002; Ferreira et al. 2006). FH is able to discriminate between host and non-host structures by binding via the domain 20 to sialic acids or glycosaminoglycans on host cells and simultaneously via the domain 19 on C3b leaving the regulatory domains free (Kajander et al. 2011). A third binding site for C3b has been located within FH8-18 (Sharma & Pangburn 1996; Jokiranta et al. 2000; Jokiranta 2006). Interactions with heparin are mapped to the domains 7 and 20 (Pangburn et al. 1991; Blackmore et al. 1996; Blackmore et al. 1998) and a possibly weaker interaction within the domain 9 (Ormsby et al. 2006). Moreover, FH16-20 has been shown to interact with sialic acids on the surface of *Neisseria* (Ram et al. 1998; Lewis et al. 2012) and it has also been proposed, based on binding studies of tryptic cleavage fragments of FH to polyanions, that the domain 13 of FH binds polyanions/sialic acids (Pangburn et al. 1991).

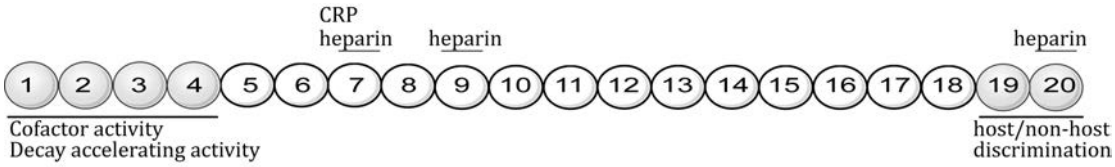

Figure 6. Presentation of the elongated FH-molecule. The main C3b-binding sites are located in the N-terminal domains 1-4 and domains 19-20 (shaded domains) and heparin binding sites are on the domains 7, 9, and 20 as indicated. The domains for surface recognition are located in the C-terminus (FH19-20).

Mutations in FH molecule are associated with renal diseases such as atypical hemolytic syndrome (aHUS) and dense deposit disease (DDD) (Warwicker et al. 1998; Richards et al. 2001). The majority of mutations found in aHUS are clustered within FH19-20, the binding site for the C3d part of C3b, sialic acids, and glycoasaminoglycans. The cell damage that is characteristic in aHUS occurs when mutated FH fails to recognize C3b deposited on glomerular basement membrane (Kavanagh & Goodship 2010). DDD is a disease associated with an FH deficiency or dysfunction of FH. Characteristic for pathogenesis of DDD is the inability of FH to protect glomerular basement membranes. This leads to overactivation of the AP and deposition of C3b and TP components in the glomeruli.

In 2005, five groups reported a polymorphism in the domain 7 of FH that is strongly associated with a condition called age-related macular degeneration (AMD), an eye disease causing visual loss in the elderly (Edwards et al. 2005; Hageman et al. 2005; Haines et al. 2005; Klein et al. 2005). In the risk allele a tyrosine at position 402 (402Y) in the domain 7 is mutated to a histidine (402H). This site is in the proximity to the binding site of heparin, CRP, and the M protein of *Streptococcus pyogenes* (Giannakis et al. 2003). Indeed, the Y402H polymorphism was shown to affect binding of FH to heparin and surface glycosaminoglycans (Clark et al. 2006; Prosser et al. 2007). The FH(402H) allotype showed an impaired binding to CRP partly explaining the inflammation in macula of patients with AMD (Laine et al. 2007).

FH-like protein 1 (FHL-1) is an alternatively spliced protein from the same gene as FH. This protein consists of seven domains identical to the N-terminus of FH followed by an unique tail of four amino acids (Ripoche et al. 1988). As expected on the basis of its similarity to the N-terminus of FH, it has one binding site for C3b and cofactor as well as

decay accelerating activities within FHL1-4, but lacks the target recognition capacity (Kühn et al. 1995; Kühn & Zipfel 1996).

FH-related proteins (FHRs) are encoded by different genes next to the gene coding for FH (and FHL-1) and share structural similarity with FH (Diaz-Guillen et al. 1999). There are five FHR proteins circulating in plasma and based on their conserved domains and ability to form dimers, FHRs can be divided into two groups (Skerka et al. 2013). Group I consists of FHR1, FHR2, and FHR5, which is characterized by a highly conserved N-terminus. These proteins are present in plasma exclusively as dimers (Goicoechea de Jorge et al. 2013; Tortajada et al. 2013). Group II includes FHR3 and FHR4 which lack the N-terminal dimerization motif in the N-terminus domains. All five FHR proteins show high homology to the C-terminal domain of FH and can probably discriminate between self and nonself surfaces similarly to FH (Hellwage et al. 1999; McRae et al. 2005; Heinen et al. 2009; Eberhardt et al. 2013; Goicoechea de Jorge et al. 2013; Tortajada et al. 2013). FHR1 has been suggested to bind C5 and inhibit the function of C5 convertase and the formation of MAC (Heinen et al. 2009). FHR2 was previously described as a C3b and heparin binding protein and recently was suggested to inhibit C3 convertase mediated cleavage of C3 (Eberhardt et al. 2013). Both FHR3 and FHR5 have been found to have cofactor activity for FI cleavage of C3b but this has not been confirmed.

FHR1 has two different forms, FHR1α (37 kDa) and FHR1β (42 kDa), dependent on glycosylation (Timmann et al. 1991). The FHR4 gene encodes two proteins, FHR4A (86 kDa) and FHR4B (42 kDa) (Jozsi et al. 2005). FHR4 binds to C3b and cofactor activity is reported for both FHR4A and FHR4B (Hellwage et al. 1999; Hebecker et al. 2010).

Properdin stabilizes both AP C3 and C5 convertases by decreasing their natural decay. Thus it is the only positive regulator in the complement activation system (Fearon & Austen 1975b; Medicus et al. 1976a; Alcorlo et al. 2013). Moreover, there are indications that properdin acts as a pattern recognition molecule binding to negatively charged molecules on apoptotic and necrotic cells and on surfaces of microbes such as *Neisseria gonorrhoeae* and *Chlamydophila pneumoniae* (formerly *Chlamydia pneumoniae*) (Spitzer et al. 2007; Cortes et al. 2011). Bound to the surface, properdin controls C3b deposition and assembly of C3bBb thereby amplifying complement activation (Spitzer et al. 2007). Individuals deficient in properdin are more susceptible to recurrent *Neisseria* infections (Densen 1989).

Vitronectin, originally called S-protein, is involved in regulation of the TP. It binds the fluid phase C5b-7 complex preventing it from binding to a nearby surface (Podack et al. 1978a; Podack et al. 1984). In addition to its role in complement regulation, it has a crucial role in cell adhesion, migration, and angiogenesis (Preissner & Seiffert 1998). In plasma, vitronectin exists either as a single chain molecule (84 kDa) or as a two-chain molecule with the chains linked together by disulfide bonds (69 and 15 kDa).

Clusterin (SP-40, 40, Apolipoprotein J). In a similar fashion as vitronectin, clusterin interacts with the C5b-7 complex and inhibits its attachment to cell membranes (Murphy et al. 1989). Moreover, clusterin binds the terminal components C7, C8, and C9. Although its role in the regulation of MAC is unclear, the interaction with C9 suggests inhibition of formation of the transmembrane pore (Tschopp et al. 1993). Clusterin has a function in clearing of cell debris and lipid transportation (Jenne & Tschopp 1992).

Factor I (C3b-inactivator) is a serine protease that degrades both C3b and C4b in the presence of a cofactor. Cleavage of C3b occurs at three specific sites resulting in the fragments C3a, C3b, C3c, and C3dg (Harrison & Lachmann 1980; Medof et al. 1982a). The two first cleavages are assisted by FH, CR1, or MCP, and results in inactivation of C3b to iC3b and release of the small nonfunctional C3f fragment (Pangburn et al. 1977; Fearon 1979; Seya et al. 1986). Further cleavage of iC3b by factor I (CR1 as a cofactor) results in the release of C3c-molecule in the fluid phase leaving the C3dg-fragment attached to the cell surface. Under physiological conditions the third cleavage only takes place in the presence of CR1 as a cofactor (Ross & Lambris 1982). C3b and iC3b attached on membranes are major opsonic fragments that can be recognized by receptors on phagocytic cells. Therefore, it is crucial that iC3b is cleaved into the non-opsonic C3dg on host cells (van Lookeren Campagne et al. 2007).

Degradation of C4b is supported by C4BP, MCP, or CR1 (Iida & Nussenzweig 1981; Seya et al. 1986). Factor I cleaves the α-chain of C4b at two sites releasing a large C4c fragment and leaving the smaller C4d attached on the membrane.

The plasma concentration of factor I is on average 35 μg/ml but it increases during inflammation since it is an acute phase protein. It consists of a heavy (50 kDa) and a light chain (38 kDa) held together with disulphide bonds. The C3b-binding site is located in the heavy chain while the protease activity is in the light chain (Goldberger et al. 1987; DiScipio 1992). Deficiency of factor I can lead to recurrent infections of encapsulated bacteria such as *Neisseria meningitidis*, *Haemophilus influenzae*, and

Streptococcus pneumoniae (Vyse et al. 1994; Nita et al. 2011; Alba-Dominguez et al. 2012).

2.2.3.2 MEMBRANE BOUND REGULATORS

Decay accelerating factor (DAF, CD55) accelerates the decay of AP and CP C3 convertases. It binds to membrane bound C3b or C4b and inactivates the convertase by dissociating the Bb- or C2a- fragments from the convertases (Nicholson-Weller et al. 1981). Two isoforms of DAF are known, a membrane-bound and a soluble form (Medof et al. 1982b; Medof et al. 1987a). The membrane bound protein is attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor, followed by a serine/threonine/proline (STP) -rich region and four SCR domains (Medof, *et al*., 1987). The functional sites within DAF are located within domains 2-4 for C3bBb and domains 2-3 for C4b2a, respectively (Brodbeck et al. 1996). Recently, two novel membrane bound isoforms of DAF were identified (Vainer et al. 2013).

Membrane cofactor protein (MCP, CD46) is expressed ubiquitously on the surface of nucleated cells and functions as a cofactor in the factor I-mediated cleavages of C4b and the first cleavage of C3b (Seya et al. 1986; Seya et al. 1988). It has structural similarity with DAF with four SCR domains and a highly glycosylated STP-region, but it lacks the GPI-anchor. Instead, it consists of a transmembrane region followed by a cytoplasmic domain containing a signaling motif. The STP- and cytoplasmic regions are alternatively spliced resulting in several isoforms that can be expressed on the same cell (Post et al. 1991). Crucial domains for the regulatory activity are located to SCR2-4 and the domains 3 and 4 are important for the cofactor activity (Adams et al. 1991).

CD59 (Protectin) is abundantly expressed on all cells types (Meri et al. 1990b). It binds to the membrane bound C5b-8 and prevents incorporation of C9 to the complex thereby blocking the formation of MAC (Meri et al. 1990a). Similarly to DAF, CD59 is a GPI-anchored protein (Davies et al. 1989). A somatic mutation in hematopoietic stem cells can affect synthesis of the GPI-anchor in the blood cells derived from stem cells resulting in erythrocytes deficient in CD59 and DAF. This leads to complement-mediated lysis of this subset of blood cells causing intravascular anemia and thromboembolic phenomena characteristic of paroxysmal nocturnal hemoglobinuria (PNH) (Nicholson-Weller et al. 1983).

Complement receptor 1 (CR1, CD35) is expressed on all human blood cells (except some T cells), follicular dendritic cells, and Kupffer cells (Fearon 1980; Tedder et al.

1983; Wilson et al. 1983; Fischer et al. 1986). It is involved in several important functions such as complement regulation, phagocytosis (described in 2.3), and immune complex clearance (Fearon 1979). In addition to its crucial role as a cofactor for factor I it has a decay accelerating activity on the AP C3 convertase and the C3 and C5 convertases of CP (Fearon 1979; Iida & Nussenzweig 1981). A soluble form, sCR1, is circulating in plasma at low concentration (30 ng/ml) (Yoon & Fearon 1985), and is most probably released from leukocytes by enzymatic cleavage (Hamer et al. 1998). There is also evidence that CR1 is a receptor for C1q (Klickstein et al. 1997) and MBL (Ghiran et al. 2000; Jacquet et al. 2013). The ectodomain of CR1 is organized into repeating units of seven SCRs, called long homologous repeats, and two additional SCRs at the C-terminal end. The two most common forms are composed of 30 SCRs or 37 SCRs (Dykman et al. 1984).

Complement receptor of the immunoglobulin family (CRIg) is an important complement receptor on Kupffer cells, the liver resident macrophages, where it mediates phagocytosis of C3b- or iC3b bound particles (Helmy et al. 2006). Moreover, CRIg regulates the AP by preventing the interaction of C3 and C5 with the AP convertases (Wiesmann et al. 2006).

Table 5. Membrane bound complement regulators

	kDa	Function	Reference
CR1	190-220	CA[1], DAA[2]	(Fearon 1979)
DAF	70	DAA	(Nicholson-Weller et al. 1981)
MCP	45-70	CA	(Seya et al. 1986)
CD59	20	Inhibits MAC[3]	(Meri et al. 1990b)
CRIg	44	Inhibits AP convertases	(Wiesmann et al. 2006)

[1]cofactor activity; [2]decay accelerating activity; [3]membrane attack complex

CUB and Sushi multiple domains 1 (CSMD1) is a transmembrane protein that inhibits CP and LP by promoting cleavage of the activated C3b and C4b by factor I and inhibition of MAC formation at the C7 level (Escudero-Esparza et al. 2013). Containing 29 SCR domains, the protein acts as a complement inhibitor highly expressed in central nervous system and epithelial tissues in a rat model (Kraus et al. 2006).

2.3 PHAGOCYTOSIS OF MICROBES BY NEUTROPHILS

Phagocytosis is a complex process where specialized cell types recognize and engulf foreign particles, such as microbes. Cells involved in microbial phagocytosis are macrophages, monocytes, dendritic cells, and neutrophils. Neutrophils comprise about 60% of the total leukocyte population and are crucial in defense against pathogens (Amulic et al. 2012). Furthermore, upon infection the number of neutrophils increases dramatically (Kim et al. 2008). Neutrophils have a characteristic lobulated nucleus and for that reason they are also called polymorphonuclear leukocytes (PMNs). Importantly, neutrophils are equipped with unique storage structures known as granules that contain antimicrobial compounds and are essential in elimination of the engulfed microbes. Since most of the pathogens are encountered at mucosal surfaces or in tissues, neutrophils have to migrate out of the blood vessels through the epithelium and move towards the infection site before they can phagocytose and eliminate the invader.

2.3.1 TRANSMIGRATION

At the sites of infection the bacteria-derived components such as LPS and formylated peptides (fMLP; N-formyl-Met-Leu-Phe) and host-produced factors such as cytokines (e.g. tumor necrosis factors, and interleukins) stimulate endothelial cells in the vicinity. The activated endothelial cells produce molecules that slow down the neutrophils on endothelial cells near the infection site, causing “rolling” of the neutrophils on the vessel wall (Moore et al. 1995). Next, in a process known as “firm adhesion” the neutrophils interact with the endothelial cells via binding of neutrophil β2-integrins, including complement receptor 3 (CR3), to endothelial intercellular adhesion molecule (ICAM)-1 (Diamond et al. 1990). Neutrophil rolling results in clustering of β2-integrin receptors on the neutrophils, which allows transmigration of the cells from the blood vessel and across the endothelial junctions and even transcellularly through the endothelial cells (Campbell et al. 1998; Phillipson et al. 2006). Although in some tissues other receptors and mechanisms are likely to be involved, the description given above is an overview of the general concept how neutrophils are recruited to the infection site.

2.3.2 CHEMOTAXIS AND PRIMING OF NEUTROPHILS

After the phagocytes have crossed the endothelial barrier, they encounter an excessive amount of chemotactic factors that direct them to the site of infection where inflammatory stimulants prime and activate the cells. Recognition of PAMPs by the cells (e.g. neutrophils, macrophages and dendritic cells) within the infection site induce the production of inflammatory cytokines and interferons.

The chemotactic factors are released by host cells or fragments generated after complement activation. The small complement peptides C5a, C3a, and to lesser extent C5a-desArg are produced after proteolytic cleavage of C3 and C5. The fragments bind to membrane bound receptors of the GPCR superfamily (Joost & Methner 2002). Although the receptors share high homology they have different ligand specificity and functions (Lee et al. 2001). C5a receptor ($C5a_1R$) has high affinity for C5a and more weakly for its truncated form, C5a-desArg, while $C5a_2$-receptor ($C5a_2R$, C5a receptor-like 2) binds C5a and C5a-desArg equally (Cain & Monk 2002; Okinaga et al. 2003). C3a-receptor (C3aR) recognizes C3a (Wilken et al. 1999), which is a much weaker chemotactic stimulus than C5a (Fernandez et al. 1978).

Other important GPCRs in neutrophil chemotaxis are FPRs that recognize the N-formylated proteins and peptides secreted by bacteria (Schiffmann et al. 1975; Gao et al. 1999).

The primary cellular sensors of pathogens are the Toll-like receptors (TLRs) of which ten different TLRs are identified in humans (Medzhitov 2007). TLR1, 2, 4, 5, and 6 are expressed on the cell surface and are essential in recognizing molecular patterns from bacteria, fungi and protozoa. TLR3, 7, 8, and 9 are expressed within endocytic compartments and recognize nucleic acids derived from bacteria or viruses. TRL10 is also involved in viral recognition by macrophages (Lee et al. 2014). Essential bacterial TLR ligands are LPS in the outer membrane of Gram-negative bacteria, which is recognized by TLR4 (Poltorak et al. 1998). TLR2, instead senses peptidoglycan of Gram-positive bacteria (Schwandner et al. 1999). Furthermore, the three TLRs 1, 2, and 6 recognize lipoproteins (Brightbill et al. 1999; Takeuchi et al. 2002), flagellin is the ligand for TLR5 and cytidine-phophateguanosine (CpG-)-rich bacterial DNA is recognized by TLR9 as well as TLR5, respectively (Hayashi et al. 2001). TLRs are transmembrane glycoproteins and most of them form homodimers upon ligand binding. For example, formation of the TLR2-TLR6 heterodimer recognizes diacylated lipopeptides from Gram-positive bacteria and mycoplasma (Takeuchi et al. 2001; Kang et al. 2009). Dimerization brings the cytoplasmic domains of the TLRs together leading to activation and recruitment of signaling components. Roughly, the TLR signaling is distinguished into the MyD88- and the TRIF- (TIR-domain-containing adapter-inducing interferon-β) dependent pathways. Both signaling pathways eventually result in activation of cells depending one or more transcription factors leading to generation of inflammatory cytokines and chemokines.

2.3.3 OPSONIZATION AND PHAGOCYTOSIS

Opsonization with complement components, immunoglobulins, or other innate immune factors is crucial for neutrophil phagocytosis. Pathogens that are covered with complement cleavage products are recognized by complement receptors 1, 2, 3, and 4 (CR1, CR2, CR3, CR4), or CRIg. Two receptors, CR1 and CRIg, have complement regulatory functions as well (section 2.2.3).

CR1 has the highest affinity for C3b followed by C4b and iC3b (Ross et al. 1983; Medof & Nussenzweig 1984). The number of CR1 molecules expressed on erythrocytes is low (average 500 per cell), however, since these cells are abundant in blood the number in blood exceeds that on leukocytes. Immune complexes (IC) are transported into the liver and spleen for phagocytosis where adenosine triphosphate (ATP) released from erythrocytes is suggested to have a stimulatory effect on phagocytosis of immune complexes (Melhorn et al. 2013). Release of the complex occurs when C3b is degraded to iC3b which has lower affinity for CR1 (Medicus et al. 1983) and higher for CR3 and CR4 allowing erythrocytes to return to the circulation (Nelson 1953; Medof et al. 1982b; Schifferli et al. 1986) leaving immune complexes bound to the spleen or liver cells. Resting neutrophils have approximately 5000 CR1 molecules per cell, and CR1 expression is rapidly up-regulated by C5a-desArg, fMLP, and increased temperatures (Fearon & Collins 1983; Berger et al. 1984). Opsonization facilitates phagocytosis of pathogens by stimulating the CR3 and Fc-receptors (Ehlenberger & Nussenzweig 1977). Moreover, neutrophils release large amounts of sCR1 when stimulated with fMLP and TNF-α (Danielsson et al. 1994) suggesting a physiological role for unbound CR1. The highest level of CR1 is expressed on B cells (20,000-40,000 per cell) (Fearon & Collins 1983). The high number may be needed for the factor I to cleave C3b to generate C3dg and binding to the nearby CR2 on B cells.

CR2 (CD21) is composed of 15 or 16 SCRs, a transmembrane domain, and a short cytoplasmic tail. It has a key function in B cell activation and in bridging innate and adaptive immune systems. CR2 has a high affinity for C3dg and iC3b fragments whereas a lower affinity for C3b and C3(H_2O) (Ross et al. 1983; van den Elsen & Isenman 2011). In B cell activation the antigen is recognized by B cell receptor (BCR) while the C3dg attached to the antigen is recognized by CR2. Crosslinking of BCR and CR2 leads to coupling of the co-receptors CD19 and CD81. Upon crosslinking, the cytoplasmic tail of CD19 is phosphorylated and a downstream signaling cascade is activated enhancing the B cell activation (Fearon & Carter 1995). In a mouse model, C3dg was shown to act as an adjuvant, increasing the immunogenicity of an analysed antigen 1,000 to 10,000 fold,

depending on the number of C3dg fragments bound to the antigen (Dempsey et al. 1996).

CR3 (CD11b/CD18, Mac-1) is a β2-integrin receptor expressed on neutrophils, monocytes, macrophages, dendritic cells, and natural killer cells (Underhill & Ozinsky 2002). It is a heterodimer consisting of a unique α-subunit (CD11b) and a non-covalently associated β subunit (CD18) (Corbi et al. 1988; Larson & Springer 1990). The ligands for CR3 are iC3b and C3b and this interaction has an important function in phagocytosis.

CR4 (CD11c/CD18) is a β2-integrin, similar to CR3, and it differs from that receptor only in its α-chain, CD11c. It is mainly expressed on tissue-derived macrophages where it has the same ligands and functions as CR3 (Myones et al. 1988).

Immunoglobulins contribute to elimination of a pathogen in many ways. Via binding to microbial epitopes they prevent spreading by immobilization (agglutination), block microbial attachment to host cells (neutralization), and mediate opsonization directly and by activating the CP. IgM and IgG1, IgG3 classes and subclasses, respectively, are effective in activation of the CP and in neutralization of bacterial toxins and virulence factors (Walport 2001). The major opsonins IgG3 and IgG1 are recognized by FcγRs on neutrophils. These receptors are divided into two classes, the activating and inhibitory FcγRs (Daeron 1997), and expression of the IgG subclasses determines the threshold for phagocytosis of the IgG-opsonized particles. Furthermore, on the basis of structural and biochemical differences the activating receptors are divided into three, FcγRI, FcγRII, and FcγRIII. The subclass FcγRIIa is the major receptor for phagocytosis on neutrophils (van de Winkel & Capel 1993). Upon cross-linking by binding to a ligand the CRs and FcγRs induce uptake of the pathogen and the plasma membrane of the neutrophil extends around its target and creates a vacuole known as a phagosome (Nordenfelt & Tapper 2011). Neutrophils are extremely efficient and can phagocyte an IgG-opsonized target within 20 seconds (Segal et al. 1980).

2.3.4 ELIMINATION OF MICROBES

Neutrophils are equipped with antimicrobial molecules that efficiently kill pathogens. When a pathogen is phagocytosed, the assembly of a nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase is induced and high amounts of ROS are generated leading to oxidative burst (Borregaard & Cowland 1997; Babior 1999; Leto & Geiszt 2006). In addition, a variety of toxic antimicrobial compounds are stored within granules that are

utilized when microbes are phagocytosed. In a calcium dependent manner the granules are fused with the phagosome where the content is released (Jaconi et al. 1990; Nordenfelt et al. 2009) and the microbe is exposed to a high concentration of antimicrobial peptides. The pH inside the phagosomes of neutrophils is close to neutral (Jankowski et al. 2002), whereas in macrophages an acidic pH is maintained (Claus et al. 1998).

Neutrophils use also a strategy to trap the microbes by expelling their nuclear, granular and cytosolic content forming a sticky mesh, called neutrophil extracellular traps (NETs) (Brinkmann et al. 2004). Most likely NETs are important in the bloodstream where the antimicrobial protein concentration is too low for efficient elimination of microbes. However, when associated with NETs the antimicrobial proteins could reach their critical concentration for efficient microbial killing.

2.4 MICROBIAL EVASION OF COMPLEMENT AND PHAGOCYTES

An invading microbe is quickly recognized as a target for elimination by the immune system in a healthy person. However, microbes have evolved various mechanisms to escape from targeted killing. Since the complement and phagocytes are the major components of the first line of defense, survival of the microbes in the human body depends on efficient evasion strategies.

2.4.1 MICROBIAL COMPLEMENT EVASION

The tightly regulated complement cascade involves several enzymes, protein complexes and receptors that provide many sites that could be interfered by microbial molecules.

A variety of microbes produce a capsule consisting of loosely attached polysaccharides to mask their surface antigens, thereby preventing AP activation in the absence of anti-capsular antibodies. Examples of well-known capsulated pathogenic bacteria are some types or strains of the Gram-negative bacteria *Escherichia coli* (Bortolussi et al. 1983; Corbett & Roberts 2009), *Haemophilus influenzae,* and *Neisseria meningitidis* as well as the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus pneumoniae* (Watson et al. 1995).

C3 is a key player in the complement cascade and therefore an ideal target for inhibition by microbes. Several proteins from *S. aureus* are shown to directly interact with the C3- or C5- molecules through various mechanisms and are discussed in the section 2.5.1.

Other microbes known to bind C3 include *S. pneumoniae* via the choline-binding pneumococcal surface protein A (PspA) (Cheng et al. 2000), *Moraxella catarrhalis* via ubiquitous surface protein A (UspA) (Hallström et al. 2011), *Candida albicans* via pH-regulated antigen 1 (Pra1) (Luo et al. 2010), *Yersinia enterocolitica* via *Yersinia* adhesion A protein (YadA) (Schindler et al. 2012). Interestingly, herpes simplex virus 1 (HSV-1) and the flagellate *Trypanosoma cruzi* trypomastigotes express proteins with decay accelerating activity on C3bBb (Fries et al. 1986).

Several microbes acquire proteases or protease precursors from plasma. Plasminogen is circulating in the blood as an inactive precursor of the serine protease plasmin that dissolves fibrin clots. In addition, plasmin binds and degrades C3 (Barthel et al. 2012). An increasing number of microbes have been shown to recruit plasminogen or plasmin onto their surfaces by various receptors. *Streptococcus pyogenes* has an enzyme that binds and cleaves plasminogen to active plasmin which subsequently cleaves IgG and C3b, leading to impaired recognition of the targets by the complement and FcγR on phagocytes (Lähteenmäki et al. 2001). Secretion of proteins that inactivate complement proteins is a common mechanism among microbes. Recently, the protease NaIP from *N. meningitidis* was shown to degrade C3 into a longer form of C3b that resulted in reduced C3b-deposition on the bacterial surface (Del Tordello et al. 2014). Other bacterial proteases that target the C3 molecule include *S. pyogenes* SpeB (Terao et al. 2008), and a few *Pseudomonas aeruginosa* proteases (Hong & Ghebrehiwet 1992; Schmidtchen et al. 2003). The *E. coli* protease EspP cleaves C3 and C5 (Orth et al. 2010), while the yeast secreted aspartyl proteases (Saps) of *C. albicans* and the surface protease PgtE of *Salmonella enterica* degrades C3b, C4b, and C5 (Ramu et al. 2007; Gropp et al. 2009).

Host cells are protected by the presence of complement regulators on their surfaces, either the membrane-bound regulators or soluble regulators that have been acquired onto the host cell surface. Most pathogens do not express regulators but have evolved means to protect themselves from complement attack by acquisition of complement regulators onto their surfaces. Tables 6 and 7 give a summary of microbes that have been reported to bind host complement regulators.

Table 6. Microbial acquisition of FH

Microbe	Ligand	Reference
Actinobacillus actinomycetemcomitans	Omp100	(Asakawa et al. 2003)
Aspergillus fumigatus	?	(Behnsen et al. 2008)
Borrelia burgdorferi s.l.	BbCRASP[1]	(Kraiczy et al. 2001; Hartmann et al. 2006)
Borrelia burgdorferi s.l.	OspE	(Hellwage, *et al.,* 2001)
Borrelia hermsii	FhbA[1]	(Hovis, *et al.,* 2006)
Borrelia recurrentis	HcpA[2]	(Grosskinsky, *et al.,* 2009)
Candida albicans	Gpm1p[1]	(Poltermann, *et al.,* 2007)
Candida albicans	Pra1[1]	(Luo, *et al.,* 2009)
Fusobacterium necrophorum	?	(Friberg, *et al.,* 2008)
Haemophilus influenzae, Hib	?[1,2]	(Hallström, *et al.,* 2008)
Haemophilus influenzae, NTHi	P5	(Rosadini, *et al.,* 2014)
Loa loa	?	(Haapasalo, *et al.,* 2008*)*
Neisseria gonorrhoeae	Por1A[1,2]	(Ram, *et al.,* 1998)
Neisseria gonorrhoeae	Por1B[1,2]	(Ngampasutadol, *et al.,* 2008)
Neisseria meningitidis	Fhbp	(Schneider, *et al.,* 2009)
Neisseria meningitidis	NspA	(Lewis, *et al.,* 2010)
Neisseria meningitidis	PorB2	(Lewis, *et al.,* 2013)
Onchocerca volvulus	?	(Meri, *et al.,* 2002)
Pseudomonas aeruginosa	Tuf[2]	(Kunert, *et al*., 2007)
Rickettsia conorii	OmpB	(Riley, *et al.,* 2012)
Salmonella enterica	Rck	(Ho, *et al.,* 2010)
Streptococcus agalactiae	β protein	(Areschoug et al. 2002)
Streptococcus agalactiae	SHT	(Maruvada et al. 2009)
Streptococcus pneumoniae	Hic	(Jarva, *et al.,* 2004)
Streptococcus pneumoniae	PspC	(Hammerschmidt, *et al.,* 2007)
Streptococcus pyogenes	Fba[1]	(Pandiripally, *et al*., 2002)
Streptococcus pyogenes	M protein[1]	Horstmann et al., 1988
Streptococcus pyogenes	Scl[2]	(Reuter, *et al.,* 2010)
Streptococcus suis	Fhb	(Pian, *et al.,* 2012)
Treponema denticola	?	(McDowell, *et al.*, 2009)
Yersinia enterocolitica	Ail	(Biedzka-Sarek, *et al.,* 2008)
Yersinia enterocolitica	YadA	(China, *et al.,* 1993)

[1]Binds also FHL-1, [2]Binds also FHR1
Abbreviations: Ail, attachment-invasion locus protein; BbCRASP, *Borrelia burgdorferi* complement regulator-acquiring surface proteins; Fba, fibronectin-binding protein; FhbA, factor H-binding protein; fHbp, factor H binding protein; Gpm, phosphoglycerate mutase; Hcp, human complement and plasminogen binding protein; Hic, factor H-binding inhibitor of complement; Nsp, neisserial surface protein; Omp, outer membrane protein; Osp, outer surface protein; Por, porin; Pra, pH-regulated antigen; Psp, pneumococcal surface protein; Rck, resistace to complement killing; Scl, streptococcal collagen-like protein; SHT, streptococcal histidine trial; Tuf, elongation factor Tu; Yad, *Yersinia* adhesin
* non-typeable

Binding of the CP and LP regulator, C4BP, is also extensively studied and the binding is mediated via domains within the α-chain (Blom et al. 2009). Many pathogens bind C4BP with the same molecule they use for binding to FH. Examples of those are Por1A of *N. gonorrhoeae* (Ram et al. 2001), Rck of *S. enterica* (Ho et al. 2011), YadA and Ail of *Y. enterocolitica* (Kirjavainen et al. 2008), and Pra1 of *C. albicans* (Luo et al. 2011). C4BP is furthermore bound by *B. pertussis* FHA (Berggård et al. 1997), *M. catarrhalis* UspA (Nordström et al. 2004), *N. meningitidis* PorA (Jarva et al. 2005), *E. coli K1* OmpA

(Prasadarao et al. 2002), *Borrelia recurrentis* CihC (Grosskinsky et al. 2010), as well as several *S. pyogenes* M-proteins (Thern et al. 1995).

The long and still growing list of pathogens binding FH and C4BP emphasizes the importance of the mechanism in microbial immune evasion. Other soluble regulators, such as C1INH, vitronectin, clusterin, and factor I are also described being used by some microbes (Table 2). Membrane-bound regulators are used by only a limited number of pathogens but some use them for entry into the host cells. CR1s on erythrocytes are utilized by *Plasmodium falciparum* (Spadafora et al. 2010; Tham et al. 2010) and *Leishmania major* (Da Silva et al. 1989). Coxackie A 21 virus (Shafren et al. 1997), Echovirus 7 (Ward et al. 1994), and Dr fimbriated *E. coli* (Nowicki et al. 1993) use DAF whereas MCP are used by measles virus for invasion (Naniche et al. 1993; Manchester et al. 2000).

Table 7. Microbial acquisition of complement regulators others than FH

Protein	Microbe	Ligand	Reference
C1INH	*Bordetella pertussis*	Vag8	(Marr, *et al.,* 2007, Marr, *et al.,* 2011)
	Borrelia recurrentis	CihC	(Grosskinsky, *et al.,* 2010)
	Escherichia coli	StcE	(Lathem et al. 2004)
C4BP	*Bordetella pertussis*	FHA	(Berggård, *et al.,* 1997)
	Borrelia burgdorferi s.l.	?	(Pietikäinen et al. 2010)
	Borrelia recurrentis	CihC	(Grosskinsky et al. 2010)
	Candida albicans	Pra1	(Luo, *et al.,* 2011)
	Escherichia coli	OmpA	(Prasadarao, *et al.,* 2002)
	Moraxella catarrhalis	UspA	(Nordström, *et al.,* 2004)
	Neisseria gonorrhoeae	Por1A	(Ram, *et al.,* 2001)
	Neisseria meningitidis	PorA	(Jarva, *et al.,* 2005)
	Porphyromonas gingivalis	?	(Potempa, *et al.,* 2008)
	Salmonella enterica	Rck	(Ho, *et al.,* 2011)
	Staphylococcus aureus		(Hair, *et al.,* 2012)
	Streptococcus pneumoniae	Enolase	(Agarwal, *et al.,* 2012)
	Streptococcus pyogenes	M prot.,	(Thern, *et al.,* 1995)
		protein H	(Ermert et al. 2013)
	Yersinia enterocolitica	YadA, Ail	(Kirjavainen et al. 2008)
	Yersinia pseudotuberculosis	Ail	(Ho, *et al.,* 2012)
Clusterin	*Staphylococcus aureus*	?	(Partridge et al. 1996) (Chhatwal et al. 1987)
	Staphylococcus epidermis	?	(Li & Ljungh 2001)
	Streptococcus pyogenes	SIC	(Chhatwal et al. 1987; Åkesson et al. 1996)
	Escherichia	?	(Chhatwal, *et al.,* 1987)
Factor I	*Staphylococcus aureus*	ClfA	(Hair et al. 2008)
	Prevotella intermedia	?	(Malm et al. 2012)
CR1	*Plasmodium falciparum*	PfRh4	(Spadafora et al. 2010; Tham et al. 2010)
CD59	*Escherichia coli*	?	(Rautemaa et al. 1998)
	Helicobacter pylori	?	(Rautemaa et al. 2001)
MCP	*Streptococcus pyogenes*	M protein	(Okada et al. 1995)
Vitronectin	*Haemophilus influenzae,* Hib	Hsf	(Hallström et al. 2006)
	Moraxella catarrhalis	UspA2	(Singh et al. 2010)
	Neisseria meningitidis	Msf	(Griffiths et al. 2011)
	Rickettsia conorii	Adr1	(Riley et al. 2013)
	Streptococcus pneumoniae	PspC	(Voss et al. 2013)

Abbreviations: Adr , adhesion of *Rickettsia*; Ail, attachment-invasion locus protein; Cih, complement inhibition; Clf; clumping factor; CRASP, complement regulator-acquiring protein; FHA, filamentous hemagglutinin; Hsf, *Haemophilus* surface fibrils; Msf, Meningococcal surface fibrils; Omp, outer membrane protein; PfRh, reticulocyte-binding-like homolog protein; Por, porin; Pra, pH-regulated antigen; Psp, pneumococcal surface protein; Rck, resistance to complement killing; SIC, streptococcal inhibitor of complement; Stc, secreted protease of C1 esterase inhibitor from EHEC; Usp, ubiquitous surface protein; Yad, *Yersinia* adhesin

2.4.2 MICROBIAL EVASION OF PHAGOCYTOSIS

For a microbe, preventing detection by phagocytes is critical for avoiding phagocytosis and subsequent destruction. As described in the section 2.3, phagocytes recognize microbes mainly via PRRs, CRs and FcγRs. For these receptors to identify the target the phagocyte needs to find the microbe and this is mediated by chemotaxis. Microbes can inhibit any of these phenomena and, in addition, microbes may directly inhibit phagocytes by causing damage to these host cells.

2.4.2.1 EVASION OF RECEPTOR-MEDIATED RECOGNITION

In addition to blocking deposition of opsonic complement components on their surface, microbes have been reported to use some other strategies to inhibit complement-mediated phagocytosis. Secreted microbial proteins can directly bind to CR3 and block the chemotactic and phagocytic functions of neutrophils. The β-glucan surface on the yeast *C. albicans* is mainly recognized by CR3 on neutrophils (van Bruggen et al. 2009). However, the secreted protein Pra1 interacts with CR3 and inhibits the uptake and subsequent phagocytosis of the pathogen (Soloviev et al. 2007; Jawhara et al. 2012). Interestingly, since CR3 recognizes FH, some FH-binding microbes have been suggested to utilize the FH-CR3 interaction for their own internalization into CR3 expressing epithelial cells, such as *N. gonorrhoeae* and *S. pneumonia* (Agarwal et al. 2010). Some microbial leukotoxins bind to β2-integrins (CR3 or leukocyte function antigen-1; LFA-1) on neutrophils and manipulate the cellular downstream. These include the RTX (repeat in toxin) family cytotoxins of Gram-negative genera, such as the *Bordetella*, *Escherichia*, *Moraxella*, and *Vibrio* (Welch 2001).

In the presence of antibodies, most pathogenic strains of *S. pyogenes,* produce the proteases IdeS, SpeB, and EndoS that target and cleave the surface bound IgG, thereby hindering neutrophil recognition (Collin & Olsen 2001). Secretory IgA (SIgA) interacts with FcγRI expressed on phagocytes and strains of the mucosal bacteria *Neisseria, S. pneumoniae,* and *H. influenzae* express IgA proteases (Plaut et al. 1975; Male 1979; Murphy et al. 2011). Recently, EsiB (*E. coli* SIgA binding protein) from extraintestinal *E. coli* was found to bind SIgA leading to impaired FcγRI cross-linking and further downstream signaling. As a result, neutrophil chemotaxis was inhibited as well as the activation of the NADPH-oxidase (Pastorello et al. 2013).

Upon release of bacterial and host-derived chemotactic fragments, phagocytes move towards the infection site. However, many pathogens have evolved ways to impair this

recruitment. The endopeptidase ScpA of *S. pyogenes* cleaves C5a and a dehydrogenase binds and inactivates C5a (Terao et al. 2006).

2.4.2.2 OTHER EVASION MECHANISMS

Toxins that form pores on neutrophils and subsequently lyse the cells are important players in innate immune evasion, such as streptolysin S found on *S. pyogenes* (Miyoshi-Akiyama et al. 2005). Gram-negative bacteria have evolved type III secretion systems (T3SS) that allow direct injection of bacterial proteins into the cytosol of the neutrophil to disrupt the neutrophil signaling network and the production of ROS. Examples include the *Yersinia* outer proteins (Yops) (Ruckdeschel et al. 1996; Diepold et al. 2010), and exotoxins of *Pseudomonas* and *Salmonella* (Geddes et al. 2007). Several other microbes have mechanisms which downregulate the production of ROS, like *C. albicans* (Wellington et al. 2009). Moreover, individuals with defects in the assembly of the NADPH-oxidase are susceptible to recurrent infections of *S. aureus*, *C. albicans* and *Aspergillus spp.* (Segal 1996).

After phagocytic uptake the microbe faces an array of bactericidal mechanisms which several pathogens manipulate for their own advantage. *Francisella tularensis* does not trigger an oxidative burst, likely by disturbing intracellular signaling (Sandström et al. 1988). *Helicobacter pylori* blocks NADPH-oxidase by binding to the phagosome thereby redirecting the granules to the plasma membrane leading to extracellular ROS release (Allen et al. 2005). Intracellular fate of bacteria within neutrophils is not as well characterized as compared to macrophages, however, *S. pyogenes* and *N. gonohorreae* resist killing by delaying the fusion of granules to the phagosome which may contribute to the persistence in the host (Staali et al. 2006; Johnson & Criss 2013).

Escape from the NET formation is described for a few pathogens. The polysaccharide capsule, together with a modulation of its lipoteichoic acids, protects *S. pneumonia*e against elimination by NETs (Wartha et al. 2007). *H. influenzae* entrapped in biofilms, which consists of both host derived DNA from NETs and bacteria-derived DNA, is resistant to both NET killing and elimination by phagocytes recruited to the site of infection (Thornton et al. 2013). Another mechanism utilized by *S. pyogenes* and *S. pneumoniae* is the production of DNAses (Sumby et al. 2005; Buchanan et al. 2006).

2.5 EXAMPLES OF INNATE IMMUNE EVASION

2.5.1 *STAPYLOCOCCUS AUREUS* – IMMUNE EVASION STRATEGIES OF A GRAM-POSITIVE BACTERIUM

Ever since Ogston identified *S. aureus* as one of the first bacterial pathogens in 1880 this bacterium has remained a major human pathogen (Smith 1982), causing a variety of diseases such as superficial and deep skin infections and more severe conditions including abscesses, sepsis, and endocarditis.

S. aureus naturally colonizes the mucosal membranes of the nose and the skin of 20-30% of the human population (Lowy 1998). Skin and soft tissue infections (SSTI) may occur if there is a wound or another lesion in the skin allowing the bacteria to enter. A previous infection does not generate protective immune responses and recurrence of SSTI is not uncommon. Usually the infections are mild but in a number of cases the infection leads to life-threatening disease (Lowy 1998). In Europe *S. aureus* has been isolated from 10% of the hospital-acquired bloodstream infections (Fluit et al. 2001). More seldom, *S. aureus* is the causative agent for respiratory, bone, and joint infections.

Some staphylococcal diseases result from effects of a specific toxin, such as staphylococcal food poisoning, staphylococcal scalded skin syndrome, and toxic shock syndrome. For example, toxic shock syndrome is initiated with the localized growth of toxin-producing strains following systemic effects of the absorbed and blood circulation distributed superantigen toxin; the fatality rate is high unless appropriate antibiotics are rapidly administered (DeVries et al. 2011).

2.5.1.1 CELL WALL

The ability of *S. aureus* to cause infection is due to its various cell surface proteins as well the secreted ones. Moreover, several strains are covered with a polysaccharide capsule and an additional “slime-layer” consisting of monosaccharides, proteins and peptides. The cell wall is built up of two major parts, a peptidoglycan layer and teichoic acid. The peptidoglycan layer consists of glycans with two alternating sugars, *N*-acetylmuramic acid (MurNAc) and *N*-acetylglucosamine (GlcNAc), which form a disaccharide unit. Peptide subunits of *L*- and *D*- amino acids are linked to the *N*-acetylmuramic acid and the peptide subunits are highly cross-linked by interpeptide bridges that make the cell wall rigid (Amako et al. 1982). Host lysozyme naturally breaks the bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine aiming at killing the bacteria, but a staphylococcal enzyme which causes *O*-acetylation of the

peptidoglycan makes *S. aureus* resistant to lysis by lysozyme (Bera et al. 2005). The other major component of the cell wall, the teichoic acids, are phosphate-containing polymers covalently bound to the peptidoglycan layer (wall teichoic acids) or to membrane-bound lipids (lipoteichoic acids). Modified phosphatidylglycerol with *D*-lysine has been shown to protect *S. aureus* against antimicrobial peptides produced by the host (Peschel et al. 1999; Collins et al. 2002b). By modulating the composition and charge of the lipoteichoic and the cells wall teichoic acids and phospholipids in the cell membrane, *S. aureus* protects itself against defensins and cathelicidins generated by the host (Peschel 2002).

2.5.1.2 ESCAPE FROM OPSONIZATION

The majority of the staphylococcal surface proteins serve as adhesins and invasins but some are clearly a part of the innate immune evasion machinery (Fig. 7, modified from Spaan *et al*, 2013). Staphylococcal protein A (SpA) prevents CP activation via binding to the C1q molecule (Forsgren & Sjöquist 1966; Sjöquist & Stalenheim 1969). More importantly, SpA also interacts with the Fc region of IgG thereby inhibiting recognition by FcγR on neutrophils (Palmqvist et al. 2005). By binding to Fab regions on IgM it cross-links the B cell receptor and modulates downstream signals and inhibits the adaptive immune responses against staphylococcal antigens (Falugi et al. 2013). Recently, SpA was shown to be released from the bacterial wall suggesting that it has a broader role and functions similar to secreted peptides (Becker et al. 2014).

S. aureus clumping factor A (ClfA) interacts not only with fibrinogen and fibrin but also factor I leading to enhanced degradation of C3b (Cunnion et al. 2004; Hair et al. 2008). Staphylococcal binder of IgG (Sbi) has also a membrane bound and a secreted form and both forms have been suggested to contribute to immune evasion (Smith et al. 2011). The N-terminal part of the molecule binds the IgG Fc region similar to SpA and apolipoprotein H (Zhang et al. 1998; Atkins et al. 2008), and the C-terminus interacts with the C3 molecule (Haupt et al. 2008).

In addition to Sbi, *S. aureus* has two other groups or families of secreted proteins that interact with C3, the SCIN- (staphylococcal complement inhibitor) and the Efb- (extracellular fibrinogen binding protein) protein families. The SCIN-family consists of SCIN-A, -B, and -C all of which share 46-48% sequence identity with each other (Rooijakkers et al. 2005b; Jongerius et al. 2007). A fourth SCIN protein, SCIN-D (ORF-D), has also been identified but it does not interact with any of the C3 fragments and a clear function for it is currently not known (Rooijakkers et al. 2007). SCIN proteins target C3

convertases of both AP and CP/LP and stabilize the convertases on bacterial surface and in a fluid phase, thereby slowing down generation of new convertases (Rooijakkers et al. 2005b; Jongerius et al. 2007; Rooijakkers et al. 2009). It was shown that dimerization of SCIN-A in a complex, $(C3bBb/SCIN\text{-}A)_2$, was important for its full functions (Jongerius et al. 2010b). A mutant SCIN-A was able to block the convertase activity equally to the wild type but had no effect on the recognition of C3b by CR1 and CRIg, while the wild type disrupted this recognition.

Originally, Efb was identified as a 16.8 kDa fibrinogen binding protein (Boden & Flock 1994), and later it was found that its C-terminal fragment (Efb-C) interacts with C3, C3b, and C3d (Lee et al. 2004; Hammel et al. 2007b; Jongerius et al. 2007). A nearby gene was independently found by two groups to encode a 9.8 kDa protein with 44% sequence homology to Efb-C (Hammel et al. 2007b; Jongerius et al. 2007). Due to its binding activities for C3, C3b, and C3d, it was named extracellular complement binding protein (Ecb), but it is also known as Efb homologous protein (Ehp). Both proteins were shown to contribute to pathogenesis of *S. aureus* infections *in vivo* in a rat wound model or in various mouse models (Mamo et al. 1994; Palma et al. 1996; Jongerius et al. 2012). The Ecb and Efb proteins block C3b-containing convertases leading to decreased production of C5a (Jongerius, *et al*., 2007). A molecular explanation for this event was suggested when Ecb was shown to enhance the interaction between FB and C3b, suggesting stabilization of the C3b-FB complex, since cleavage of FB to its Bb and Ba fragments was impaired (Jongerius, *et al*., 2010). However, contrasting results were reported in another study with Efb-C, which inhibited initial FB binding to immobilized C3b (Chen et al. 2010). The binding between C3d and Efb-C/Ecb also disrupts the C3d-CR2 interaction (Ricklin et al. 2008), which has potentially functional consequences for B cell activation and adaptive immune responses.

Also, Sbi and Efb are shown to recruit plasminogen for degradation of C3 and C3b *S. aureus* has several molecules that can proteolytically cleave complement components. The secreted staphylokinase (SAK) targets plasminogen to the bacterial surface, which is subsequently cleaved into plasmin (Lähteenmäki et al. 2001). Plasmin cleaves the opsonins IgG, C3b, and iC3b leading to impaired neutrophil phagocytosis (Rooijakkers et al. 2005a). Also, Sbi and Efb are shown to recruit plasminogen for degradation of C3 and C3b (Koch et al. 2012). The metalloprotease aureolysin cleaves C3 and subsequently blocks all complement pathways (Laarman et al. 2011). *S. aureus* has also been shown to acquire the regulator FH via SdrE (Sharp et al. 2012) and via a Sbi-C3b complex (Haupt et al. 2008) resulting in decreased iC3b conversion.

S. aureus has several "staphylococcal superantigen-like proteins" (SSL). One of them SSL-10, interacts with IgG (Itoh et al. 2010) while SSL-7 binds human IgA (Langley et al. 2005) leading to impaired interaction of IgG and IgA with FcγRI on neutrophils. Moreover, SSL-7 binds to and prevents the cleavage of C5 (Bestebroer et al. 2010; Laursen et al. 2010). All the above mentioned mechanisms lead to impaired neutrophil uptake.

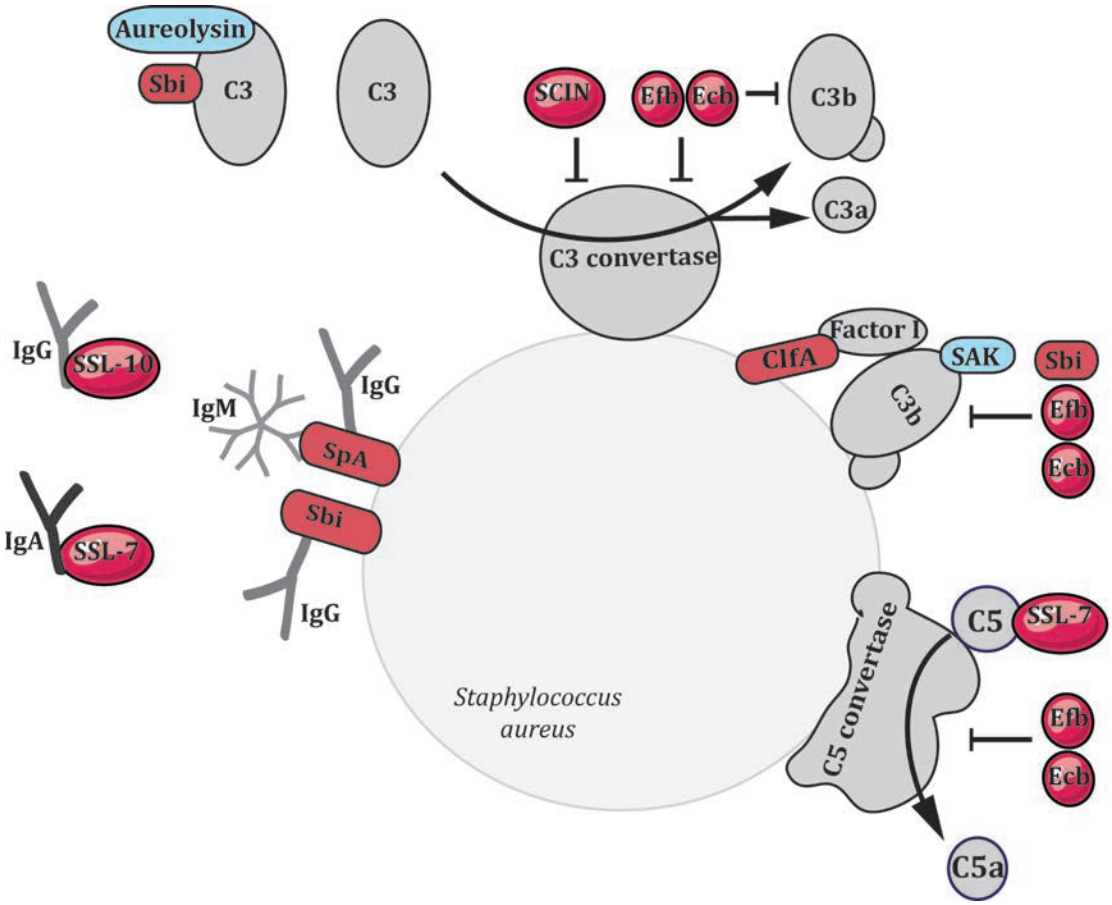

Figure 7. *S. aureus* escape from complement and opsonization. Proteins in dark grey indicate host's complement proteins. Membrane- bound or secreted virulence proteins marked in red are binding to and blocking the functions of complement components or immunoglobulins. The proteins in blue are proteases that degrade complement components. Abbreviations: Clf, clumping factor; Ecb, extracellular complement binding protein; Efb, extracellular fibrinogen binding protein; SAK, staphylokinase; Sbi, staphylococcal IgG-binding protein; SpA, staphylococcal protein A; SSL, staphylococcal superantigen-like protein. (Picture modified from (Spaan et al. 2013).

2.5.1.4 ESCAPE FROM NEUTROPHILS AND NETS

The *S. aureus* SSL-5, SSL-11, and extracellular adherence protein (Eap) interfere with ligands required for neutrophils to roll and adhere onto endothelial cells (Chavakis et al. 2002; Bestebroer et al. 2010). SSL-5 is also an inhibitor of chemokine receptors as well as C5aR (Bestebroer et al. 2009). Staphylococcal chemotaxis inhibitory protein (CHIPS) blocks the C5aR as well as FPRs on neutrophils and several other (FPR)-like inhibitory proteins are shown to bind to FPR. This eventually results in impaired phagocytic responses (de Haas et al. 2004; Prat et al. 2006; Kretschmer et al. 2010). Secretion of the protease Staphopain A inhibits the CXC chemokine receptor-2 (CXCR2) dependent chemotaxis and activation (Laarman et al. 2012). *S. aureus* also has mechanisms that directly eliminate neutrophils. Secreted cytolytic toxins, such as the two-component leukocidins, attack the cell membrane resulting in leakage and lysis of the cells. For example, the Panton-Valentine leukocidin is present in most strains causing community-acquired methicillin-resistant *S. aureus* (MRSA) infections (Naimi et al. 2003). A schematic overview of molecules involved in escape from neutrophils is shown in Fig. 8 (figure modified from Spaan *et al*., 2013). In addition, there is evidence that *S. aureus* can survive inside host cells (Thwaites & Gant 2011), as well as lysing the neutrophils after phagocytosis (Kobayashi et al. 2010).

S. aureus escapes from NETs by secreting the two staphylococcal nucleases staphylococcal nuclease (Nuc) (Berends et al. 2010) and adenosine synthase A (AdsA) (Thammavongsa et al. 2009; Thammavongsa et al. 2011) that degrade the DNA. The golden pigment staphyloxanthin is an antioxidant that protects against ROS (Liu et al. 2005). Other molecules contributing to *S. aureus* resistance against ROS are catalase that converts hydrogen peroxide to oxygen and water (Mandell 1975), and SOK (surface factor promoting resistance to oxidative killing) (Liu et al. 2005).

The large number of molecules that act and inhibit distinct stages of the innate immunity as well as factors that form a bridge between innate and adaptive immunity has turned *S. aureus* into a “master of immune evasion”. On top of all the virulence mechanisms, *S. aureus* is effective in acquiring antibiotic resistance and the emergence of resistant strains has become a global problem (DeLeo et al. 2010) which offer challenges for the future.

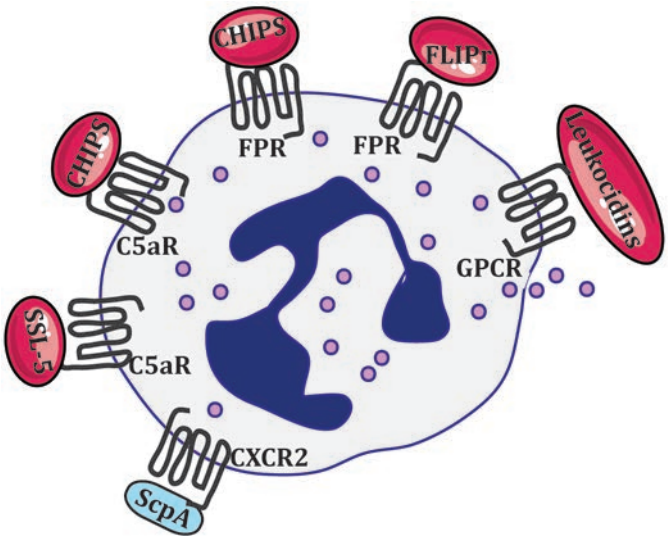

Figure 8. *S. aureus* escape from neutrophils. Molecules in the red ovals are *S. aureus* factors that can bind and block the functions of the receptors. The protein in the blue oval is a protease that cleaves the N-terminal of CXCR2. Abbreviations: C5aR, C5a receptor; CHIPS, chemotaxis inhibitory protein of *staphylococcus;* CXCR2, chemokine receptor; GPCR, G-protein coupled receptor; FPR, formyl protein receptor; FLIPr, FPR-like inhibitory proteins; ScpA, staphopain A; SSL, staphylococcal superantigen-like (Figure modified from Spaan *et al*., 2013).

2.5.2 *BORDETELLA PERTUSSIS* – IMMUNE EVASION STRATEGIES OF A GRAM-NEGATIVE BACTERIUM

Bordetella pertussis is the causative agent of whooping cough (pertussis, "hundred day cough") and was first isolated in 1906 by Bordet and Gengou (Mattoo & Cherry 2005). The highly contagious bacteria colonize the ciliated epithelial cells of the upper respiratory tract in humans. Symptoms include paroxysmal coughing with whooping, often followed by post-coughing vomiting. Coughing can persist for weeks or even months, but in individuals with partial immunity the symptoms are likely to be milder (Cherry et al. 2004).

The earliest vaccines that consisted of killed whole *B. pertussis* cells brought the epidemic under control but they were associated with severe adverse events (Cody et al. 1981). Today these vaccines are mostly replaced by the second generation vaccines

containing one to five purified bacterial components being filamentous hemagglutinin (FHA), pertactin, two types of fimbriae, and detoxified pertussis toxin (Ptx) (see section 1.5.2.3). Protection after vaccination varies depending on the quality of the vaccine and immunization schedule in use and immunity, but may last from 4-12 years (Lugauer et al. 2002; Wendelboe et al. 2005). The vaccine coverage in the population is high, in Finland as high as 98% (Mertsola 2001), which keeps the disease under control. In spite of this the circulation of strains in the community has not decreased.

2.5.2.1 LIPOPOLYSACCHARIDE

The complex LPS (endotoxin) of Gram-negative bacteria consists of lipids covalently linked to polysaccharides comprising the inner and outer core regions. The core regions may be linked to an *O*-side chain (*O*-antigen) which is composed repetitive glycomers (Caroff & Karibian 2003). *B. pertussis* lacks the *O*-chain (Peppler 1984; Burns et al. 2003) and is suggested to have an advantage in complement evasion (Pishko et al. 2003). Lipid A is a part of the outer leaflet of the outer membrane and anchors the core oligosaccharide to the cell wall. The LPS of *B. pertussis* appears as two distinct bands, A and B, in a silver stained SDS-gel (Peppler 1984). Band B of the core oligosaccharide contains the sugars heptose and glucose, and glucuronic acid, glucosamine, and galactosaminuronic acid (GalNAcA) (Caroff et al. 1990). The slower migrating band A consists of band B connected to a trisaccharide of *N*-acetyl-*N*-methylfucosamine (FucNAcMe), 2,3-deoxy-di-*N*-acetylmannosaminuronic acid (2,3-diNAcManA), and *N*-acetylglucosamine (GlcNAc) (Caroff et al. 1990). In general, the lipid A on LPS is recognized and opsonized by surfactant protein A secreted by alveolar cells in the lungs. The *B. pertussis* LPS, however, has a structure that protects against surfactant protein A, possibly by steric hindrance (Schaeffer et al. 2004). This leads to destabilization of the bacterial outer membrane, aggregation of bacteria thereby hindering adhesion to alveolar epithelia, and promotion of phagocytosis (Van Iwaarden et al. 1994).

2.5.2.2 VIRULENCE FACTORS

Expression of most virulence factors of *B. pertussis* are regulated by the two-component control system, BvgAS. BvgAS is a transmembrane sensor protein that upon environmental stimulation controls the three phenotypic phases. The Bvg^+ phase expresses molecules needed during colonization and invasion while during the Bvg^- phase the phenotype is avirulent. It is unclear whether the avirulent phenotype is functional in *B. pertussis*. A third intermediate phase, Bvg^i, is suggested to have an essential function in the transmission and early respiratory tract colonization

(Stockbauer et al. 2001). Important *B. pertussis* virulence factors are schematically described below and presented in figure 9.

Colonization. The first step for a mucosal pathogen is to adhere to the epithelial cells and *B. pertussis* expresses various proteins that facilitate the colonization of the respiratory tract. The major adhesins are FHA, fimbriae (Relman et al. 1989; van den Berg et al. 1999; Rodriguez et al. 2006), and pertactin (Leininger et al. 1991). FHA is a large (232 kDa) protein present as surface-attached and secreted forms (Renauld-Mongenie et al. 1996). Its C-terminal domain is required for colonization of the respiratory tract in a rat model (Julio et al. 2009). The long, thin structures of fimbriae extend from the cell membrane and contain heparin-binding regions, which most likely are involved in the adherence (Geuijen et al. 1998). The fimbriae consist of one of the major subunits, Fim2 or Fim3, and an additional FimD subunit (Willems et al. 1993; Geuijen et al. 1997). Pertactin is an autotransporter protein which is characterized by an ability to direct its own secretion across the outer membrane (Leininger et al. 1991). It contains one RGD (Arg-Gly-Asp) domain that most likely is involved in adhesion. Another virulence molecule in the autotransporter family is the tracheal colonization factor (Tcf). Most likely it has an important function in colonization of *B. pertussis*, although the mechanism is still unclear (Finn et al. 1991; Finn & Stevens 1995).

Formation of biofilms is a rather complex and not extensively characterized phenomenon in *B. pertussis*. It is, however, important for mucosal pathogens by facilitating adherence and protection against antimicrobial agents and host immune defenses (Conover et al. 2010; Serra et al. 2011).

Escape from opsonization and phagocytosis. The proteins Vag8 (Finn & Amsbaugh 1998) and FHA interact with the CP of complement by binding C1INH (Marr et al. 2007; Marr et al. 2011) and C4BP (Berggård et al. 1997; Berggard et al. 2001), respectively. Moreover, the *Bordetella* resistance to killing protein (BrkA) mediates complement resistance by inhibiting deposition of C3b, C4b, and formation of the MAC (Fernandez & Weiss 1994; Barnes & Weiss 2001). The pertactin protein, which has 29% homology with BrkA, did not contribute to serum resistance (Fernandez & Weiss 1998). However, colonization with the related animal pathogen *B. bronchiseptica* in a mouse model showed that a strain lacking pertactin inhibited clearance by neutrophils (Nicholson et al. 2009; Inatsuka et al. 2010).

Toxins are important in *B. pertussis* pathogenesis and most of them have functions in inhibition of neutrophil recruitment. Ptx is produced exclusively by *B. pertussis* among

the genus *Bordetellae*. It is composed of five subunits, S1-S5 (Tamura et al. 1982) of which the S1 subunit (A subunit) maintains the enzymatic activity. The A subunit is bound to a ring-like structure formed by the S2-S5 subunits (B oligomer) (Carbonetti 2010). Ptx binds to host cells via its B oligomer and enters the cytosol by endocytosis where it targets the GPCR resulting in modulation of the signaling pathway and intoxication of the cell (Katada et al. 1983). By binding to epithelial cells in the respiratory tract Ptx inhibits chemokine release from the cells leading to impaired chemotaxis and neutrophil recruitment (Thomazzi et al. 1995; Kirimanjeswara et al. 2005; Andreasen & Carbonetti 2009). Furthermore, *in vivo* studies have shown that Ptx can suppress the antibody responses to several *B. pertussis* antigens (Mielcarek et al. 1998; Carbonetti et al. 2004).

Tracheal cytotoxin (TCT) is a part of the cell wall of Gram-negative bacteria and while other bacteria, such as *E. coli*, recycle the disaccharide-tetrapeptide by transporting it back to its cell cytoplasm (Jacobs et al. 1995), *B. pertussis* releases it into the environment (Rosenthal et al. 1987). TCT damages the airway epithelial cells (Wilson et al. 1991). In addition, it inhibits neutrophil migration toward formylated peptides (Cundell et al. 1994). Adenylate cyclase toxin (ACT) is secreted, however, most of it interacts with FHA and remains associated with the bacterial surface (Gray et al. 2004). It quickly forms inactive aggregates in solution and a close contact with the target cell is needed for its activity. ACT inhibits neutrophil phagocytosis by increasing cyclic adenosine monophosphate (cAMP) cellular levels leading to actin cytoskeletal rearrangements and subsequently inhibition of chemotaxis and formation of ROS (Friedman et al. 1987; Kamanova et al. 2008).

In vitro studies have demonstrated that both IgG and IgA are important in neutrophil phagocytosis of *B. pertussis* (Hellwig et al. 2001a; Rodriguez et al. 2006) and *in vivo* studies showed that the neutrophil FcγR is important in the clearance of the bacteria from the lungs (Hellwig et al. 2001b; Andreasen & Carbonetti 2008). In the absence of antibodies the bacteria can be bound via a FHA-CR3 interaction and subsequently phagocytosed by neutrophils, macrophages and monocytes. Interestingly, this process does not seem to promote an oxidative burst (Berton et al. 1992) and suggest that *B. pertussis* exploits CR3 for uptake and intracellular survival. Furthermore, both Ptx and FHA enhance the expression of CR3 by neutrophils (Mobberley-Schuman & Weiss 2005) providing further evidence that uptake via CR3 is advantageous for *B. pertussis.*

2.5.2.3 OTHER BORDETELLA SPECIES

In addition to *B. pertussis,* the genus *Bordetella* includes eight other species, which most likely originate from a common environmental *B. petrii*-ancestor. The *B. pertussis* and *B. parapertussis* species have evolved as human specific pathogens from *B. bronchiseptica*-like anchestors and together these three species are known as the classical *Bordetella.* A small portion of pertussis is caused by *B. parapertussis*, and sporadically also by *B. bronchiseptica* and *B. holmesii,* the two latter ones mostly isolated from immunocompromised individuals (Woolfrey & Moody 1991; Yih et al. 1999). Other members of the *Bordetella* reported to be isolated from immunocompromised patients are *B. hinzii* (Vandamme et al. 1995), *B. avium* (Harrington et al. 2009), *B. trematum* (Vandamme et al. 1996), and *B. ansorpii* (Fry et al. 2007).

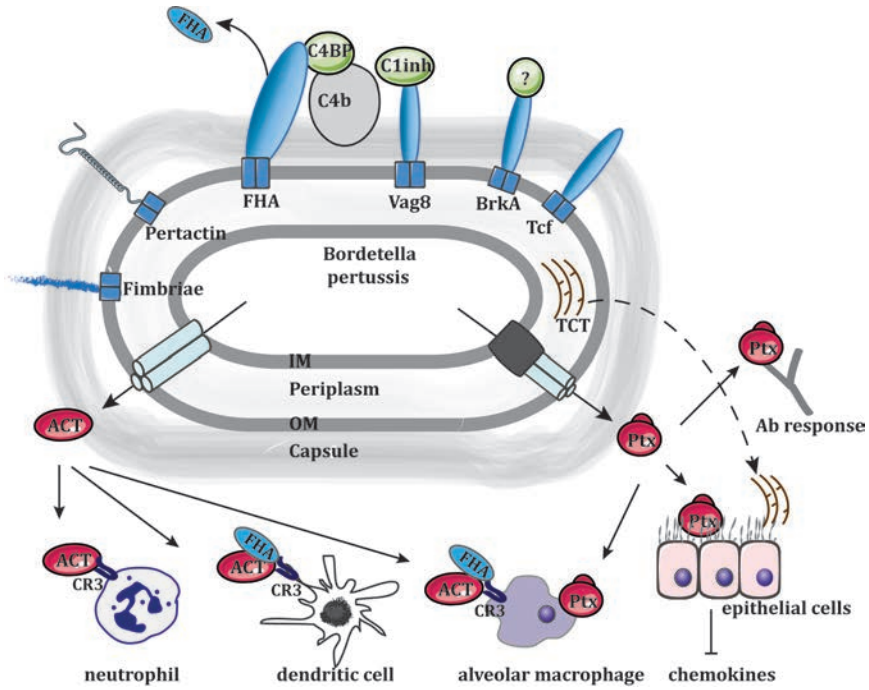

Figure 9. Presentation of important *B. pertussis* virulence factors. The adhesins, fimbriae, pertactin, FHA, Vag8, BrkA, and Tcf are shown in blue; host molecules in green; the toxins ACT and Ptx in red and TCT in brown. FHA and Vag8 bind the classical pathway regulators C4BP and C1INH, respectively, and BrkA inhibits complement opsonization by an unknown mechanism. Ptx stimulates the expression of chemokines from epithelial cells resulting in inhibited recruitment of phagocytes, including neutrophils and alveolar macrophages. Ptx directly intoxicate alveolar macrophages, and in the bloodstream it suppresses antibody responses to *B. pertussis* antigens. ACT together with FHA induces apoptosis of macrophages in a CR3- dependent manner. They block CR3 on dendritic cells and ACT bound to phagocytes modulate the signaling leading to inhibited chemotaxis and superoxide production. TCT is released from the peptidoglycan in periplasmic space and released into the extracellular environment by an unknown mechanism. It attacks and damages ciliated cells. Abbreviations: Ab, antibody; ACT, adenylate cyclase toxin; Brk, Bordetella resistance to complement; C4BP, C4b binding protein; C1INH, C1 inhibitor; FHA, filamentous hemagglutinin; IM, inner membrane; OM, outer membrane; Ptx, pertussis toxin; Tcf, tracheal colonization factor; TCT, tracheal cytotoxin.

3 AIMS OF THE STUDY

I. To study how the important pathogen *Bordetella pertussis* evades the attack by complement alternative pathway.

II. To analyze the molecular mechanisms of how and why various microbes bind complement alternative pathway inhibitor factor H via the C-terminus.

III. To determine how *Staphylococcus aureus* prevents the attack by alternative complement pathway attack.

4 MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 COMPLEMENT PROTEINS

FH and C3 were isolated from plasma of healthy laboratory workers according to Koistinen et al. (Koistinen et al. 1989) with minor modifications described elsewhere (Haapasalo, *et al.,* 2008) or obtained from Calbiochem (La Jolla, CA). C3b was prepared from C3 as described in the section 4.2.1. The C3d protein was expressed in *E.coli* (Nagar et al. 1998) and was a kind gift from Professor David E. Isenman (University of Toronto, Canada). Factors B and D were purchased from CompTech (Tyler, TX) and Factor I from Calbiochem/MerckMillipore (Darmstadt, Germany). Soluble CR1 was obtained from CellDexTherapeutics (Needham, MA).

The genes encoding recombinant proteins FH1-5, 1-6, 1-7, 8-11, 11-15, and 15-20 were cloned and the recombinant proteins produced in the baculovirus expression system described earlier (Kühn & Zipfel 1995). The genes encoding recombinant fragments FH5-7 and FH5-7 variant (FH5-7_{var}) with an Y402H substitution, were cloned and the gene products produced as described by Haapasalo, *et al.* (2008). Cloning of the gene and purification of FH19-20 was described previously (Jokiranta, *et al.,* 2006). Generation of the FH19-20 substitutions D1119G/Q1139A, D1119G, Q1139A, L1189R, E1198A, R1215Q, and W1157A, R1182A, W1183L, T1184R, K1186A, K1188A, R1203A, R1206A, and R1210A were described earlier (Lehtinen et al. 2009). The constructs with several point mutations in the domain 19 (FH19^{del}-20, with substitutions Q1137A, Q1139A, and Y1142A), or the domain 20 (FH19-20 ^{del}, with substitutions T1184G, K1202A, R1203A, and Y1205A) have been described (Kajander, *et al*., 2011). FH1-4 was expressed in *Pichia pastoris* by MSc S. Hyvärinen as described (Blanc et al. 2012).

4.1.2 BACTERIAL PROTEINS

The genes encoding outer surface proteins OspE and OspA were cloned from *B. burgdorferi* sensu stricto strain N40 and the recombinant protein was purified (Hellwage et al. 2001). The gene encoding FhbA was cloned from *B. hermsii* strain MAN (Hovis et al. 2006) and Tuf gene was cloned from a *P. aeruginosa* blood isolate strain, and the recombinant proteins were purified as previously described (Kunert et al. 2007). The genes encoding *S. aureus* proteins Ecb, Ecb$_{N63E/R75E/N82E}$, and Efb were cloned, expressed and the recombinant proteins purified as described earlier (Jongerius et al. 2007; Hammel et al. 2007; Jongerius et al. 2010).

4.1.3 OTHER PROTEINS AND SERA

Bovine and human serum albumin (BSA and HSA, respectively), gelatin and heparin were purchased from Sigma Aldrich (St. Louis, MO). Normal human serum (NHS) was obtained from at least five healthy persons belonging to the laboratory personnel and the NHS was stored at -70°C until used. To inactivate complement, NHS was heat-inactivated at 56°C for 30 min (HIS). NHS deficient in FHR-1 was obtained from an individual known to be FHR-1 deficient. For serum survival assays in the study I, NHS with IgG titer <1500 was used. The classical and lectin pathways were blocked with ethylene glycol tetra-acetic acid (EGTA) and $MgCl_2$ added to NHS to final concentrations of 10 mM and 5 mM, respectively (MgEGTA-NHS). C3 depleted serum was purchased from Quidel (San Diego, CA).

4.1.4 ANTIBODIES

Antibodies used in the thesis are listed in Table 8.

Table 8. Primary and secondary antibodies used in the studies

Antibody	Description	Supplier	Study
Primary antibodies			
Anti-FH	Goat pAb[1]	Calbiochem, CA	I
Anti-FH	Goat pAb	Quidel, San Diego, CA	III
Anti-C3c	Rabbit pAb	DAKO, Denmark	IV
Anti-C3d	Rabbit pAb	DAKO	III
Anti-FB	Rabbit pAb	Hoechst-Behring, Marburg, Germany	III
Secondary antibodies			
Anti-goat	Peroxidase-conjugated	Jackson Immunoresearch, West Grove, PA	I
Anti-rabbit	Peroxidase-conjugated	Jackson Immunoresearch	III
Anti-goat	FITC[2]-conjugated	Jackson Immunoresearch	I
Anti-rabbit	Alexa Fluor® 488-labeled	Invitrogen, Eugene, OR	IV
Anti-human C3	FITC-conjugated	Protos Immunoresearch, Burlingame, CA	IV

[1]polyclonal antibody; [2] fluorescein isothiocyanate

4.1.5 MICROBES

All microbial strains used in this thesis, including their growth conditions are listed in Table 9.

Table 9. Microbial strains and growth conditions

Bacteria	Strain and relevant feature	Reference or source	Study
*B. pertussis**	Tohama I, reference strain	(Kasuga et al. 1954)	I
*B. pertussis**	B32, mutation in *PtxP*	(Black & Falkow 1987)	I
*B. pertussis**	*B.p.* 175, Clinical isolate, Fim3, Prn2, PTXS1A	The National Public Health Institute, Turku, Finland	I
*B. pertussis**	*B.p* 406, Clinical isolate, Fim2, Prn2, PTXS1A	The National Public Health Institute, Turku, Finland	I
B. parapertussis†	ATCC 15251	(Eldering & Kendrick 1952)	I
B. holmesii‡	ATCC 51541, human isolate	(Weyant et al. 1995)	I
B. avium‡	ATCC 35086, from poultry	(Hinz et al. 1978)	I
S. aureus§	Wood 46	(Jongerius et al. 2007)	III
S. aureus§	Newman	(Jongerius et al. 2012)	IV
S. aureus§	Clinical isolate from blood	HUSLAB, Helsinki, Finland	II, IV
H. influenzae¶	Clinical isolate	HUSLAB, Helsinki, Finland	III
H. influenzae¶	Clinical isolate from blood	HUSLAB, Helsinki, Finland	II
S. pneumoniae§	Blood isolate	HUSLAB, Helsinki, Finland	II
S. pyogenes§	*emm8*, non-binder of FH	Haapasalo, *et al*., 2008	II
S. pyogenes§	st369, binds FH	Haapasalo, *et al.,* 2008	I
P. aeruginosa#	Blood isolate	HUSLAB, Helsinki, Finland	II
C. albicans||	Blood isolate	HUSLAB, Helsinki, Finland	II

* Growth conditions: 36°C; on charcoal agar without blood and containing cephalexin (0.04 mg/ml) or Stainer-Scholte broth.

† *Growth conditions: 37°C; otherwise same as B. pertussis*

‡ *Growth conditions: 35°C (B. holmesii) or 37°C (B. avium), 5% CO_2, on charcoal plates without antibiotics or brain-heart infusion broth.*

§ *Growth conditions: 37°C, 5% CO_2; on blood-agar, chocolate-agar plates, or Todd-Hewitt broth*

¶ *Growth conditions: 37°C, 5% CO_2; on chocolate-agar plates or brain-heart infusion broth*

Growth conditions: *37°C, 5% CO_2; on cled plates*

|| Growth conditions: 33*°C; on Saboraud agar*

4.2 METHODS

4.2.1 GENERATION OF C3b

The C3b was generated from C3 by trypsin cleavage. Trypsin (final volume 1% w/w; Sigma Aldrich) was added to C3 in phosphate buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl; pH 7.4) mixed, and incubated in a water bath at 37°C for 2 min. The reaction was stopped by adding soy bean trypsin inhibitor (SBTI; Sigma Aldrich) at a final concentration of 2% (w/w). The generation of C3b and C3a was indicated by a shift in the size of the α-chain to α'-chain visualized after SDS-PAGE and subsequent Coomassie staining. The samples were centrifuged and proteins separated by gel filtration (HiLoad 16/60 Sephadex 200, GE Healthcare).

4.2.2 RADIOLABELING OF PROTEINS

Proteins were labeled with ^{125}I (Perkin Elmer) using the IodoGen method according to the manufacturer's instructions (Thermo Scientific Pierce, Rockford, IL). Briefly, iodogen (1,3,4,6-tetrachloro-3a,6a-diphenyl-glycoluril, approx. 1 mg/ml) (Thermo Scientific or Sigma) was mixed with chloroform (25 ml) and allocated (50 µl) into glass tubes and air-dried. The protein (20-50 µg in 100 µl of PBS) was mixed with 4 µl of ^{125}I in the coated iodogen tube and after incubation for 10 min the mixture was transferred into a glass tube. The non-bound ^{125}I was separated from bound by running the mixture through a PD-10 column (Amersham Biosciences).

4.2.3 DIRECT ^{125}I-PROTEIN BINDING ASSAYS (I, II)

After harvesting the bacteria or yeast they were washed three times with VBS (142 mM NaCl; 5 mM diethyl barbiturate, pH 7.3) or PBS. The indicated number of cells were incubated with radiolabeled protein (25,000–40,000 cpm/reaction) in VBS, or 50% PBS containing 0.1 % gelatin (GVBS and GPBS, respectively), or 0.33% VBS. Inhibitors of the interaction were analyzed by adding unlabeled protein to the reaction mixture. After 20-30 min of incubation at 37°C with agitation (1200 rpm), the mixtures were centrifuged through 20% sucrose in GVBS or GPBS to separate cell-associated and free radioactive proteins. Radioactivity in the pellet and supernatants were measured with a gammacounter (Wallac, Turku, Finland).

4.2.4 RADIOLIGAND ASSAYS (II, III, IV)

For the radioligand assays BreakApart microtiter plates (NALGE NUNC, Roskilde, Denmark) were coated with bacteria (1x10^6 CFU/well in PBS at 37°C for 12 hours) or various proteins (2-25 µg/ml in PBS at 4°C for 12-18 hours). After washing three times with PBS, the wells were blocked with 0.5% BSA in PBS, or 0.5% BSA in 50% PBS, for 60 min at 22°C. In a separate nonadherent microtiter plate (Greiner Bio One, Frickenhausen, Germany), variable concentrations of nonlabeled proteins were mixed with the radiolabeled proteins in 50% PBS or PBS containing 0.1% BSA. Mixtures were added to the wells and incubated at 37°C for 60 min. The wells were washed with PBS or 50% PBS, dried, and bound radioactivity from wells was measured with a gammacounter.

When ^{125}I-FB binding to C3b was analyzed, coating and washing buffer was VBS and blocking buffer was VBS containing 0.5% BSA. The unlabeled and labeled proteins were incubated in ½VBS-$NiCl_2$ (0.75 mM $NiCl_2$, 2.5 mM barbitural acid, 71mM NaCl).

4.2.5 COFACTOR ASSAYS FOR C3b INACTIVATION (I, II, III, IV)

Cleavage of C3b by FI was analyzed by incubating FH, FH1-4, or sCR1 (at concentrations indicated in the studies II, III, and IV) with ^{125}I-C3b (100,000 cpm/reaction) in the absence or presence of bacterial proteins at 37°C for 5-60 min. A mixture containing ^{125}I-C3b and FI served as a negative control and ^{125}I-C3b, FI and FH served as a positive control. The samples were treated with β-mercaptoethanol (Sigma-Aldrich), heated (3 min at 93°C) and run on SDS-PAGE gels, fixed with 5% acetic acid, dried, and cleavage fragments detected by autoradiography. In the studies II and IV, the intensity of the C3b α'-chain was determined with a GelEval-programme (FrogDance Software, Dundee, UK).

For detection of the cofactor activity on a bacterial surface, approximately 2x10^9 bacteria/ml were exposed to 2% HIS in GVB at 37°C for 30 min with agitation. After washing, radiolabeled C3b (50,000 cpm/reaction) and Factor I (100 ng/reaction) were added and incubated (37°C, 30 min, 1200 rpm). The supernatants were separated from the pellets and treated as described above and detected by autoradiography.

Analysis of the cofactor activity on a cell surface (study III) was performed as described previously (Jokiranta et al. 1996). Normal rabbit erythrocytes were coated with C3b by using freshly purified C3, FB, and FD. After washes, cells were incubated with Factor I (62.5 nM), FH (1.3 µM), and Ecb (11–110 nM) for 10 min at 37°C with agitation followed by treatment with 2-mercaptoethanol. Cells were centrifuged (4 min, 500 × g), and the

supernatants were run on SDS-PAGE, transferred to a nitrocellulose membrane followed by blocking with 3% nonfat milk in PBS for 60 min at 22°C. The membranes were incubated with a mixture of rabbit anti-human C3d and C3c Abs (dilution 1:2000 for both) for 17 h at 4°C, washed with PBS, and incubated further with a peroxidase-conjugated goat anti-rabbit Ab (1:5000) for 60 min at 22°C. After washing, the proteins were detected by enhanced chemiluminescence (ECL).

4.2.6 DECAY-ACCELERATION ACTIVITY OF FH (III)

Analysis of the decay accelerating activity of FH was based on a previously described protocol (Krych-Goldberg et al. 1999). Microtiter plates were coated with 5 μg/ml C3b in PBS (4°C for 18 h). After washings with VBS, wells were blocked with VBS/0.5% BSA (22°C for 60 min). The convertases were formed by incubating the coated wells with FB (4 ng) and FD (0.4 ng) in VBS-$NiCl_2$ containing 0.05% Tween (37°C for 60 min). After washing twice with VBS-$NiCl_2$, FH (0-2 nM) with either Ecb (0-2.2 nM) or buffer was added and incubated for 20 min at 22°C. The wells were washed twice and C3bBb convertases were detected with a pAb rabbit anti-FB antibody (1:5000) followed by an anti-rabbit antibody (1:5000). Bound conjugate was detected by adding *o*-phenylenediamine dihydrochloride (DAKO) and measuring absorbance at 492 nm.

4.2.7 BACTERICIDAL ASSAYS (STUDIES I, III)

Freshly harvested *Bordetella* grown to mid-log phase were washed three times with GVBS and incubated with 10% NHS, MgEGTA-NHS, or HIS at 37°C with gentle agitation. Samples were taken after 0, 15, 30 and 60 min of incubation and the complement activation was stopped on ice before diluting and plating the samples on charcoal agar. Bacterial survival was calculated as the number of viable colonies at different times, relative to baseline colony counts at the start.

The effect of Ecb in the presence or absence of FH and FH19-20 on survival of bacteria was assayed by using a serum sensitive strain of *H. influenzae*. NHS or HIS (12% in PBS) was mixed with Ecb or BSA together with FH or FH19-20. After incubation for 5 min at 37°C with continuous agitation, bacteria (31 μl of $1x10^7$ CFU/ml) in PBS were added and incubated for 10 min. Complement activation was stopped by placing the samples on ice for 2 min, diluted with PBS followed by plating onto chocolate agar plates.

4.2.8 SERUM AND PROTEIN ABSORPTION ASSAYS (I)

Bacteria were incubated with 10% HIS, HIS deficient in FHR-1, or recombinant FH fragments for 30 min at 37°C. After washing five times with PBS-Tween, the bound proteins were eluted with an acidic buffer (0.1 M glycine-HCl, pH 2.7), the supernatants were collected and pH adjusted (pH 7) with 1M Tris-HCl (pH 9.5). Aliquots of the fractions were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. Bound proteins were analyzed with a pAb anti-FH antibody and an HRP-conjugated secondary antibody and detected by ECL.

4.2.9 FLOW CYTOMETRIC ANALYSES (I, III, IV)

Washed bacteria were exposed to NHS, HIS, or C3-depleted serum (10-30%) in the presence or absence of proteins as indicated in the studies and incubated at 37°C for 20 or 30 min with vigorous agitation. After washing with PBS containing 1% BSA or 0.05% Tween-20, the cells were incubated with anti-FH pAb (1:50 or 1:300 dilution) at 37°C for 30 min with agitation, or 4°C for 60 min. After washes, bacteria were incubated with a FITC-conjugated anti-goat antibody (*Bordetella;* 1:200, 37°C for 30 min) or Alexa® fluor 488-labeled anti-goat antibody (*S. aureus;* 1:100, 4°C for 60 min) in PBS. Samples were washed and bound antibody was detected by flow cytometry (FACScan, Becton Dickinson, or CyAN™ADP, Beckman Coulter).

4.2.10 ISOLATION OF NEUTROPHILS AND RED BLOOD CELLS (IV)

Isolation of neutrophils used in binding and phagocytosis assays was performed as previously described (Troelstra et al. 1997) with minor modifications. Blood was drawn from healthy laboratory individuals into tubes containing hirudin (Roche Diagnostic, Mannheim, Germany) and diluted 1:1 with PBS. Cells were separated by centrifugation through a gradient (Histopaque® 1.119 and 1.077, Sigma Aldrich) at 320 x *g* for 20 min at 22°C. Neutrophils were collected and washed once with RPMI Media 1640 (GIBCO®) containing 0.05% HSA (RPMI-HSA). The remaining blood cells were lysed with ice-cold water and isotonic conditions were reestablished with PBS before the neutrophils were washed and diluted to a concentration of $1x10^5$-$1x10^6$ cells/ml.

Erythrocytes were isolated from whole blood anticoagulated with EDTA. After centrifugation (500 x *g*) at 4°C for 15 min, the plasma, buffy coat, and the uppermost layer of erythrocytes were removed and the cells were washed three times with PBS containing 0.5% BSA before used in the assays.

4.2.11 C3B BINDING TO NEUTROPHILS AND ERYTHROCYTES

All assays were analyzed by flow cytometry and incubations and centrifugations involving neutrophils or erythrocytes were performed at 4°C.

Neutrophils were incubated with *S. aureus* supernatant or purified bacterial proteins for 30 min, washed with RPMI-HSA and collected (1200 rpm, 5 min). After incubation with C3b (15 µg/ml, 60 min), cells were washed and incubated with FITC-labeled anti-human C3 (10 µg/ml, 60 min) before analysis.

Alternatively, C3b (100 µg/ml) was preincubated with stapylococcal proteins (0.1 or 1 µM) at 22°C for 15 min before neutrophils were added. After 60 min incubation, cells were washed with RPMI-HSA and incubated with rabbit anti-C3c (1:50, 20 min) and Alexa Fluor® 488-labeled antibody (1:100, 20 or 30 min).

A similar assay using erythrocytes was performed where the preincubation step was excluded, i.e proteins and erythrocytes were added at the same time.

4.2.12 NEUTROPHIL BINDING AND PHAGOCYTOSIS ASSAYS (IV)

S. aureus bacteria were incubated with fluorescein coupled to *N*-hydroxysuccinimide-ester (NHS-fluorescein, Thermo Scientific) in the neutrophil binding assays, or pHrhodo™ Green STP ester (Molecular probes, Eugene, OR) in phagocytosis assays according to the manufacturer's protocol. The labeled bacteria were opsonized in NHS (20%) for 15 min before bacterial proteins (0.09 or 0.9 µM) and neutrophils were added (bacteria:neutrophil ratio was approximately 20:1) in RPMI-HSA for 60 min at 37°C. The reactions were stopped by adding ice cold RPMI-HSA and neutrophils were collected by centrifugation (400 x *g*, 10 min). After washing they were fixed with 1% paraformaldehyde and subjected to flow cytometry.

To analyze the effect of Ecb and Efb on binding of neutrophils to *S. aureus* in whole blood, the bacteria were incubated (60 min at 37°C) with 450 µl of hirudin- or EDTA-anticoagulated blood in the presence of 1.6 µM of the bacterial proteins. The reaction was stopped by centrifugation as described above and red blood cells were lysed with ice-cold water and isotonic conditions restored with PBS. Cells were washed, fixed and analyzed by flow cytometry.

4.2.13 STRUCTURAL MODELING, CURVE FITTING, AND STATISTICAL ANALYSIS

Structural modeling of the OspE:FH19-20:C3b complex in study II was based on the published structures of C3b (Wu et al. 2009), C3d and FH19-20 (Jokiranta, *et al.,* 2006; Kajander, *et al.,* 2011) and was performed by MSc Arnab Bhattarcharjee under the supervision by Sakari Jokiranta (Bhattacharjee et al. 2013). In the study III, modeling of the Ecb/Efb-C:C3d:FH19-20 was based on C3d in complex with Ecb (Hammel et al. 2007b), Efb-C in complex with C3d (Hammel et al. 2007a) and FH19-20 in complex with C3d (Kajander, *et al.,* 2011). The structural illustrations were done using PyMOL software (www.pymol.org).

Curve fitting was performed using non-linear regression models implemented in GraphPad Prism®. Statistical analyzes were done using Microsoft Excel® and GraphPad Prism ®. Comparison of mean values was done using an unpaired two-tailed t-test and results were presented with mean using standard deviation to indicate the error.

5 RESULTS

5.1 COMPLEMENT EVASION OF *B. PERTUSSIS* BY FH ACQUISITION (I)

Several Gram-negative respiratory tract pathogens bind soluble complement regulators to escape the complement attack (Kunert et al. 2007; Hallström et al. 2008). The causative agent of whooping cough, *B. pertussis*, is also known to utilize the CP regulators C4BP (Berggård et al. 1997) and C1INH (Marr et al. 2011) for immune evasion, but no data on acquisition of host AP inhibitors has been reported. However, it has been previously shown that *B. pertussis* is particularly resistant to AP attack *in vivo* (Pishko et al. 2003).

We analyzed CP and AP sensitivity of seven *Bordetella* strains and found that all the *Bordetella* strains capable of infecting humans were resistant to killing by the AP *in vitro* (Fig. 1, study I). When both of the pathways were active, two *B. pertussis* clinical isolates (strains 175 and 406) were fully resistant to killing. Analysis of FH binding in a direct binding assay demonstrated that all *Bordetella* strains tested, except an avian pathogen *B. avium*, bound FH (Fig. 2A, study I). The binding ranged between 13% and 32% of the total protein offered. As a positive control we used a strain of *S. pyogenes* that is known to be a strong FH binder (Haapasalo et al. 2008). Binding of FH to *Bordetella* was confirmed in an another assay where bacteria were exposed to heat inactivated serum and binding of FH was analyzed by flow cytometry (Fig. 3, study I).

Two strong FH-binding strains, the clinical isolates of *B. pertussis* (strain 175) and *B. parapertussis* (strain 15311), were selected for further studies. Both strains bound FHL-1 and FHR1 from NHS (Fig. 4, study I). Further analysis using recombinant FH fragments demonstrated that the main binding site within FH is located in the C-terminal part of FH (FH19-20). A second, much weaker binding site within FH5-7 was demonstrated by protein absorption experiments and direct binding assays (Figs. 5 and 6, study I). Finally, we showed that FH deposited onto the surface of both the strains analysed and bacteria-deposited FH was functionally active and cleaved the bacteria-bound deposited C3b into its inactive fragment iC3b (Fig. 7). From this study we concluded that the respiratory pathogens *B. pertussis* and *B. parapertussis* utilize FH for protection from AP-mediated complement attack.

5.2 MICROBIAL COMPLEMENT EVASION BY BINDING FH19-20 (II)

The majority of the pathogens which utilize FH as an evasion mechanism bind FH either via domains 5-7, 19-20, or both, as demonstrated also for *Bordetella* in the study I. In the study II we aimed to find a plausible explanation for why various microbes bind FH via its C-terminus by investigating the binding interaction at a molecular level.

We selected five microbes that have previously been shown to bind FH via FH19-20, including the Gram-negative bacteria *P. aeruginosa* (Kunert et al. 2007), *H. influenzae* (Hallström et al. 2008), and *B. pertussis* (study I), the Gram-positive *S. pneumoniae* (Hammerschmidt et al. 2007), as well as the yeast *C. albicans* (Meri et al. 2002). All microbes were isolates from blood cultures, except for *B. pertussis* that was isolated from the nasopharynx. All the strains employed bound FH19-20 and fourteen different FH19-20 fragments containing surface-exposed point mutations were tested for their capacity to inhibit binding of FH19-20 to the microbes (Fig. 1, study II). In addition, we analyzed three nonhomologous bacterial proteins: the surface proteins OspE from *B. burgdorferi*, FhbA from *B. hermsii*, and Tuf from *P. aeruginosa* for their binding to FH19-20 and capacity to inhibit the interaction with FH19-20. We showed that only the fragments with a mutation within domain 20 decreased the binding in a statistically significant manner (Fig. 2A-C, study II). Furthermore, it was revealed that three key residues of FH (R1182A, R1203A, and R1206) were involved in the binding of FH19-20 to all the microbes and microbial proteins analyzed.

Since one binding site for heparin and glycosaminoglycans is located in the domain 20 (Blackmore et al. 1998), we hypothesized that microbes might mimic the host cell surfaces by binding FH via the heparin site on domain 20 while leaving domain 19 free for C3b interaction. We tested the effect of heparin on the interaction between FH19-20 and the microbial proteins and demonstrated that the microbial binding site overlapped only partially with the heparin binding site (Fig. 4, study II). Previously, it was shown that FH can bind the C3d part of C3b either via domain 19 or domain 20 (Kajander et al. 2011). We next asked whether the FH19-20:microbe interaction was affected by C3d and showed that C3d did not inhibit binding of FH19-20 to the microbial proteins (Fig. 4, study II). This important finding suggested that microbes can bind FH in a similar manner as host cells, *i.e.* FH19-20 can bind to the microbial surface and C3d simultaneously. This phenomenon is explained structurally by the studies of Bhattacharjee et al. (Bhattacharjee et al. 2013).

Results of the inhibition assays indicated that C3d does not block but enhances binding of FH19-20 to all the tested microbial proteins. By using OspE, which is unable to bind to C3b (Hellwage et al. 2001), it was next demonstrated that the domain 19 is essential for the C3b:FH19-20 interaction as well as for the formation of the tripartite complex C3b:FH19-20:OspE (Fig. 5, study II). The functional consequence of formation of the complex was analyzed in cofactor assays with FH using the N-terminal part of FH (FH1-4) as a negative control (Fig. 6, study II). We found an enhanced cofactor activity of FH when this was bound to the microbial protein. It was finally concluded that a conserved microbial binding site within domain 20 on FH was crucial for binding to FH and that microbial proteins enhance the FH:C3b interaction leading to a more efficient down-regulation of the complement attack.

5.3 EFFECT OF TRIPARTITE COMPLEX FORMATION ON COMPLEMENT EVASION (III)

S. aureus has several secreted C3 binding proteins. The two staphylococcal secreted proteins, extracellular complement binding protein (Ecb) and extracellular fibrinogen binding protein (Efb), bind to the same region on C3b as FH. Therefore, we asked whether the bacterial proteins affect the interaction between C3b and FH.

As has been demonstrated in the studies I and II, deposition of FH onto a microbe is an important immune evasion mechanism and the FH C-terminus plays a crucial role in this phenomenon. In the study III we found that the deposition of FH from NHS onto the surface of *S. aureus* was clearly dependent on the presence of both C3 and functional Ecb, since in the absence of either of these proteins FH deposition was not detected (Fig. 1, study III). In the binding assays using purified proteins, we found that Ecb enhanced the binding of FH to C3b and C3d and, moreover, this enhanced interaction was mediated by FH19-20 (Fig. 2, study III). From these experiments we concluded that Ecb formed tripartite complexes with C3b and FH19-20.

On the basis of the results obtained with RIA, the C3d region of C3b and the C-terminal end of FH play essential roles in the enhancement of Ecb to C3b. Therefore, we determined which site of FH19-20 is involved in the enhancement using the same sets of mutant FH19-20 fragments as in the study II. In contrast to the study II where we observed that domain 20 mediated the formation of the tripartite complex, here we demonstrated that a site on domain 19 was critical for mediating the tripartite complex formation (Fig. 3, study III). Moreover, in a structural model based on previous

structures of C3d:Ecb (Hammel et al. 2007b) and FH19-20:C3d (Kajander et al. 2011) we found no direct contact between Ecb and FH19-20, although they were located close to each other (Fig. 4, study III).

In the studies I and II, we showed examples of bacteria or bacterial proteins that acquire FH in a functional manner such that the regulatory activity in cleaving C3b to iC3b is retained. Surprisingly, the presence of Ecb only in very high concentrations led to enhanced cofactor activity in the fluid phase and on the surface of rabbit erythrocytes (Fig. 5A-C, study III). The decay accelerating activity of FH was weakly inhibited by Ecb and inhibition of FB binding to C3b by FH was somewhat enhanced in the presence of Ecb (Fig. 5D-F, study III).

Next, we analyzed whether the enhanced functions of FH by Ecb had an effect on the survival of bacteria. When serum sensitive *H. influenzae* bacteria were incubated with NHS in the presence of Ecb the survival of bacteria increased significantly (Fig. 6A, study III). The survival was further enhanced when FH or FH19-20 was added (Fig. 6B, study III). In contrast, the bacteria were killed in the presence of NHS only or NHS together with FH or FH19-20 but in the absence of Ecb. We concluded that by causing formation of the tripartite complexes, Ecb:C3b:FH, *S. aureus* enhances the FH-mediated defense against complement attack.

5.4 INHIBITION OF CR1-MEDIATED RECOGNITION BY STAPHYLOCOCCAL PROTEINS (IV)

In study III we showed that Ecb enhanced the deposition of FH onto the surface of *S. aureus* but, surprisingly, this did not result in increased cleavage of C3b into iC3b. Since iC3b is the main opsonin recognized by CR3 on phagocytes, it is clearly an advantage for *S. aureus* to avoid iC3b deposition on its surface. Nevertheless, also C3b is recognized by neutrophils via CR1. This led us to speculate that *S. aureus* produces one or several secreted molecules that are capable of inhibiting recognition of C3b by CR1.

We used staphylococcal culture supernatant in inhibition assays to study binding of C3b to neutrophils and identified Efb as a mediator of the inhibition (Fig. 1, study IV). Since the C-terminus of Efb is highly homologous with Ecb and both bind C3b (Hammel et al. 2007b; Hammel et al. 2007a; Jongerius et al. 2007), we included Ecb in our binding assays and showed that both the staphylococcal proteins inhibit C3b binding to neutrophils (Fig. 2B). Only Ecb inhibited attachment of neutrophils to *S. aureus* after

preopsonizing *S. aureus* with C3b generated during incubation in NHS (Fig. 6A, study IV). When we recreated the *in vivo* situation and incubated *S. aureus* in whole blood, both Ecb and Efb significantly inhibited binding of *S. aureus* to neutrophils (Fig. 6B, study IV).

Results from the direct binding analyses with purified proteins clearly showed that Ecb and Efb blocked the interaction of sCR1 with C3b (Fig. 3A, study IV). In the presence of FH, the binding was further enhanced (Fig. 3C-D, study IV). In the study III we had discovered that Ecb does not enhance the cofactor activity of FH and in the study IV we found that Ecb also blocked the cofactor activity of sCR1 for C3b (Fig. 4, study IV).

Erythrocytes are important for clearance of bacteria from blood and they express CR1, but not the other complement receptors on their surface. We showed that Ecb and Efb efficiently inhibit binding of C3b to erythrocytes (Fig. 5, study IV). To analyze the effect of Ecb and Efb on phagocytosis of *S. aureus* by neutrophils, we labeled the bacteria with a dye that increases in fluorescence in acidic conditions, i.e. upon phagocytosis of the bacteria. In the presence of Ecb, significantly fewer bacteria were phagocytosed while in the presence of Efb no difference was seen (Fig. 6C, study IV). In conclusion, it is clear that at least Ecb impairs recognition of C3b-opsonized *S. aureus* by CR1 on neutrophils, while the role for Efb in inhibition of phagocytosis remains unknown.

6 DISCUSSION

6.1 INNATE IMMUNE EVASION - DIFFERENCES BETWEEN GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

6.1.1 SURFACE STRUCTURES OF GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

The Gram-positive and Gram-negative classes of bacteria are primarily distinguished on the basis of the structure of their cell wall. The cell membrane of Gram-positive bacteria is surrounded by a thick wall to which the majority of cell surface proteins are anchored. In comparison, Gram-negative bacteria are composed of two cell membranes separated by a periplasmic space and the outer cell membrane is utilized as a platform for anchoring the outer proteins (Figure 10).

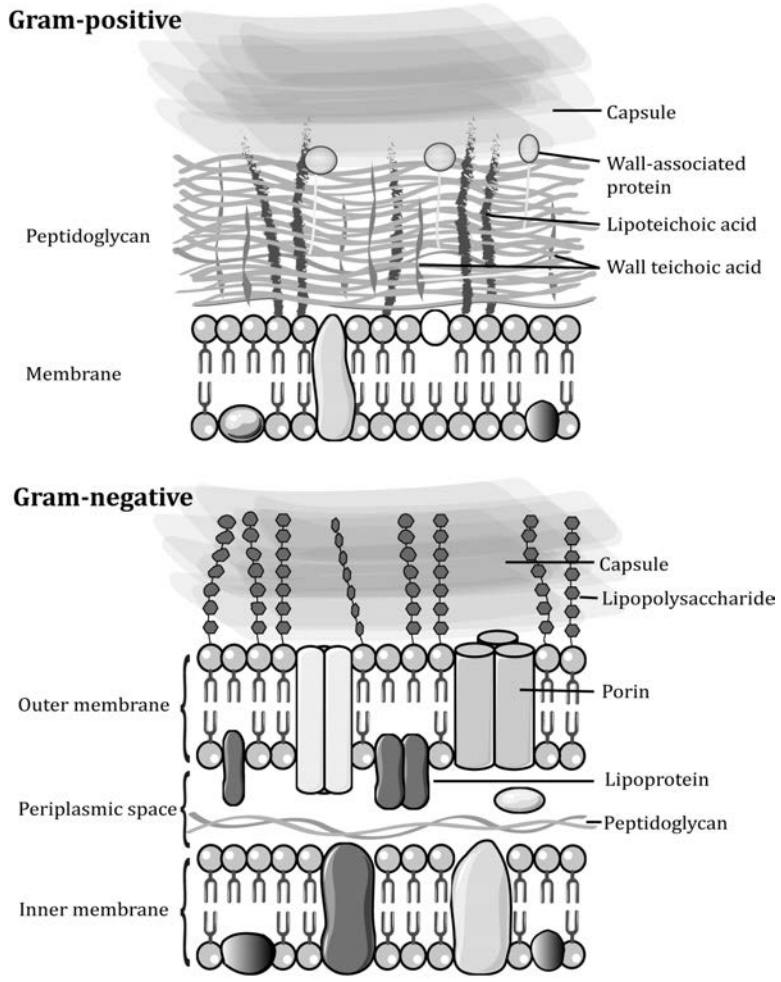

Figure 10. Schematic structures of the components of the cell walls of Gram-positive and Gram-negative bacteria.

It is generally accepted that Gram-positive bacteria are protected from complement-mediated lysis by the thick peptidoglycan layer that physically prevent the formation of MAC on the bacterial cell membrane (Joiner et al. 1984). In Gram-negative bacteria, long *O*-antigens (smooth strains) on the LPS cause steric hindrance, while rough strains with short or even without *O*-antigens are susceptible to MAC-mediated lysis (Clay et al. 2008; Goebel et al. 2008). In spite of that, the study I demonstrated that strains of *B. pertussis,* naturally lacking *O*-antigen (Burns et al. 2003), were resistant to killing by human serum. In particular, the bacteria were protected against the AP attack, which suggests that they possess factors, either on the surface or secreted, that inhibit the AP. This is in line with findings from Pishko, *et al.,* (2003) who showed that *B. pertussis* was able to survive *in vivo* by acquiring a factor from serum.

6.1.2 IMPORTANCE OF CAPSULE IN IMMUNE EVASION

The polysaccharide capsule outside the outer cell membrane is clearly important for virulence of several Gram-negative and Gram-positive bacteria, as capsule deficient strains of *S. aureus*, *S. pneumonia*, and *H. influenzae* are shown to be less well protected against complement mediated attack (Thakker et al. 1998; Hallström et al. 2006; Hyams et al. 2010). Upon cleavage of the C3-molecule a reactive thioester in C3b is exposed and reacts rapidly with hydroxyl and amine groups on the target surface (Pangburn & Müller-Eberhard 1980). Although C3b molecules are deposited on the surface of bacteria, the capsule is suggested to physically inhibit the MAC to target the membrane (Joiner et al. 1984).

When strains of *B. pertussis* are grown at 36°C, as in this study, the strains are in the virulent phase (Bvg+). During the virulent phase the capsule of *B. pertussis* is very thin and is called a microcapsule (Hot et al. 2003; Neo et al. 2010). The microcapsule did not contribute to protection against complement attack or phagocytosis and its function in pathogenesis remains elusive (Neo et al. 2010). A capsule is, however, important for optimal orientation of the membrane proteins so that they can act as immune evasion molecules.

Although a capsule seems to efficiently hinder the deposition of MAC on the bacterial membrane, several encapsulated bacteria bind the terminal pathway regulator vitronectin, for instance *H. influenzae* (Hallström et al. 2006) and some Gram-positive bacteria (Chhatwal et al. 1987). In addition, certain strains of *S. aureus* and *S. pyogenes* secrete molecules that interfere with components of the terminal pathway. For example, *S. aureus* binds C5 via its SSL7 and consequently blocks MAC assembly (Laursen et al.

2010), and *S. pyogenes* streptococcal inhibitor of complement (SIC) protein blocks the membrane-insertion site on C5b-7 inhibiting MAC formation (Åkesson et al. 1996). Recently, it was shown that C5b-9 complexes are deposited on the surface of several Gram-positive bacteria, including *S. aureus* and *S. pyogenes* (Berends et al. 2013). AP activation results in the generation of C5b-9 and eventually impaired survival of the bacteria in blood (Haapasalo et al. 2012). These findings are interesting, because they emphasize the significance of C5b-9 complexes on Gram-positive bacteria and their possible role in direct lysis by means of the terminal pathway.

6.2 COMPLEMENT EVASION AT C3 STAGE

All microbial evasion mechanisms studied in this thesis target the C3 molecule. Impairing C3 stage and downstream functions is clearly an advantage for the microbes since all three complement pathways converge on this stage.

6.2.1 CONTROL BY HOST'S SOLUBLE REGULATORS

C3b can bind to any cellular surface, including the host's own cells, therefore the regulation of complement is strongest also on human cells at the C3 level. Human cells are protected by the presence of membrane- bound regulators such as CR1, DAF, and MCF as well as by several soluble regulators. The main AP regulator, FH, regulates C3b deposition on host cell surfaces by binding C3b via the domain 19 and anionic molecules on self surfaces via the domain 20, leaving the regulatory domain free to inactivate C3b (Kajander et al. 2011).

Numerous microbes acquire FH onto their surfaces leading to generation of the inactive C3b fragment, iC3b, resulting in decreased MAC formation. Strikingly, the majority of the microbes exploit two regions for FH-binding: domains 5-7 and domains 19-20 as presented in figure 11.

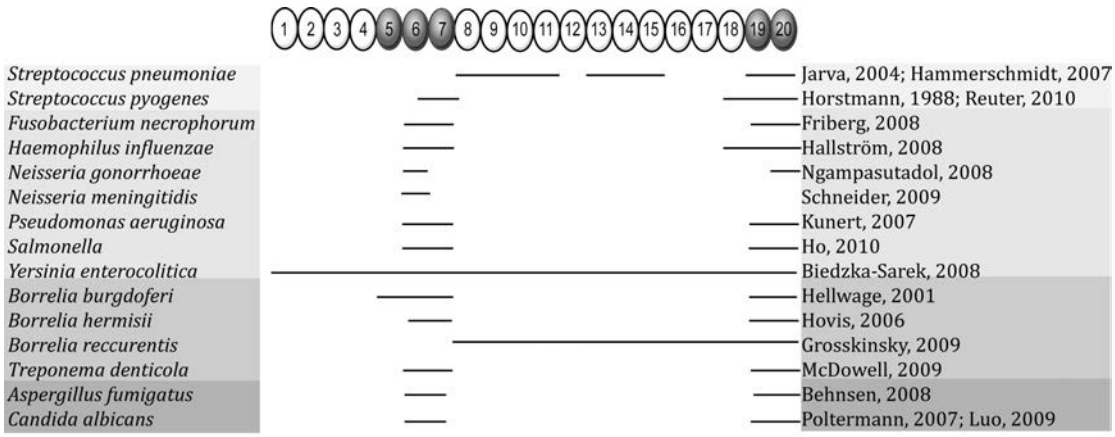

Figure 11. Microbial binding sites within FH. The identified binding sites are indicated with lines. From light to dark grey: Gram-positive bacteria, Gram-negative bacteria, Gram-negative spirochetes, and yeasts. The most important references are indicated. The figure is modified from the study II.

Depending on the microbe one of the sites may be more important for FH-binding, as demonstrated for *B. pertussis* and *B. parapertussis* in the study I where FH19-20 seemed to be essential for the binding. Despite being an important pathogen, complement evasion by *B. pertussis* has not been extensively studied. On the other hand, it can be questioned whether *B. pertussis* needs to be serum resistant since it lives on mucosal surfaces and not in blood. *Bordetella* strains such as *B. holmesii* (Tartof et al. 2014), *B. hinzii* (Cookson et al. 1994), *B. bronchiseptica* (Katzenstein et al. 1984), and *B. parapertussis* (Wallihan et al. 2013), have been reported to cause bacteremia in immunocompromised patients, but it has not been described for *B. pertussis*. Complement components are present on mucosal surfaces in the airways that are in the range 10-20% of the concentration of that in the blood (Boackle 1991; Persson et al. 1998). Therefore, respiratory pathogens such as *B. pertussis* need to protect themselves from complement.

Previous studies have demonstrated that *B. pertussis* escapes the CP (Berggård et al. 1997; Barnes & Weiss 2001; Marr et al. 2011) by binding C4bp and C1INH. A *B. pertussis* strain lacking FHA, which is the ligand for C4BP, showed no difference in serum survival as compared to the wild type strain (Fernandez & Weiss 1998). However, FHA may act in synergy with other virulence factors as described for FHA and Ptx (Relman et al. 1989) and FHA and ACT (Gray et al. 2004), which may explain the results. The study I provides important information on how *B. pertussis* and *B. parapertussis* evade the AP of

complement. Future studies need to elucidate the ligands for FH and their significance in those pathogens.

The C-terminal domains FH19-20 are central in microbial evasion but it has remained elusive how and why these domains are involved in this process. Domain 20 is utilized by host cells for discrimination between host and non-host surfaces (Lehtinen et al. 2009; Ferreira et al. 2010; Kajander et al. 2011). Therefore the hypothesis was that microbes could use a similar mechanism or even so-called molecular mimicry. Indeed, the study II revealed a conserved binding site on domain 20 used by several different microbes, but the binding site was not identical with the one that is used by the host cells. This finding was important, since an overlapping binding site reveals possibilities for designing molecules that block the microbes from binding FH without blocking the important discrimination on own cells.

Notably, the three structurally distinct proteins analyzed, OspE, FhbA, and Tuf, efficiently enhanced the cofactor activity of FH by formation of a tripartite complex consisting of C3b, FH, and microbial protein. Although a functional cofactor activity has been reported for most microbes that acquire FH, this was the first time enhanced regulation was described.

A variety of complement evasion factors have been described for *S. aureus*, but direct FH-binding has not been demonstrated. Although one study has reported FH-binding, normal human serum instead of purified protein was used in almost all the assays in that study (Sharp et al. 2012). Therefore, it was likely that other components from serum were involved. The study III gives an explanation to how *S. aureus* can utilize FH for its own protection. The secreted staphylococcal protein Ecb binds C3b using the same area as FH (Hammel et al. 2007b; Hammel et al. 2007a; Jongerius et al. 2007; Kajander et al. 2011) and our studies revealed that deposition of FH onto the surface of *S. aureus* was dependent on both C3 and Ecb. The formation of a tripartite complex between C3b, Ecb, and FH occurred, similar to what was described in the study II. In contrast to that study where domain 19 bound to C3b, here the domain 20 of FH was responsible for C3b-binding. Surprisingly, increased concentration of the *S. aureus* protein Ecb did not lead to enhanced cofactor activity as demonstrated for the proteins in the study II. The decay accelerating activity of FH, however, was slightly enhanced in the presence of Ecb. This indicates that upon formation of the Ecb:C3b:FH complex, the regulatory part of FH (FH1-4) remains free to decay the same or even a nearby convertase. The FB interaction with C3b was most likely inhibited via a conformational

change within C3b upon Ecb-binding, as previously suggested (Hammel et al. 2007b; Chen et al. 2010). The formation of the C3b:Ecb complex did, however, enhance FH binding to the complex, which in turn resulted in the inhibition of FB-C3b interaction. These results demonstrated that two out of the three functions of FH, illustrated in figure 5, were affected by the formation of a C3b:Ecb:FH complex. Increased concentration of FH significantly increased the survival of a serum sensitive strain, compared to only Ecb, and pinpointed the contribution of the enhanced functions of FH in downregulation of C3b.

6.2.2 MICROBIAL PROTEINS ACT ON C3

The evasion mechanism described in the study III revealed that increased concentration of bacterial protein Ecb did not lead to enhanced formation of iC3b, but on the contrary, blocked the generation of iC3b. This was a puzzling result, since C3b is the major ligand for CR1 expressed on most blood cells and C3b deposition eventually leads to phagocytosis. Either the C3b on bacteria is recognized by CR1 on erythrocytes and transported to the liver and spleen for elimination, or CR1 on neutrophils facilitates phagocytosis by stimulation of CR3 and CR4 (Berger et al. 1984). Moreover, the iC3b fragment has a 100-fold lower affinity for CR1 as compared to that of C3b (Kalli et al. 1991). Taken together, for *S. aureus* it seems to be beneficial to inactivate C3b into iC3b.

Therefore, the hypothesis in the study IV was that *S. aureus* has one or more secreted molecules that inhibit C3b-CR1 interaction. Indeed, both the staphylococcal proteins Ecb and the fibrinogen-binding Efb inhibited the direct C3b-CR1 protein interaction. However, only Ecb impaired the phagocytosis of *S. aureus* by neutrophils after serum exposure. When exposed to whole blood also Efb bound to neutrophils (Fig. 6B, study IV), which indicated that a factor from blood contributes to this interaction. It was recently demonstrated that the Efb:C3b complex attracts fibrinogen to the microbial surface and hinders recognition by phagocytic receptors (Ko et al. 2013), which may at least partially explain the phenomenon observed in the study IV. It is not known whether other bacteria exploit the same strategy, but another staphylococcal protein (SCIN) prevents CR1 recognition of the C3b within the convertase (Jongerius et al. 2010a).

Since the interaction site of CR1 to C3b is not identified in detail it is not clear by which mechanisms Ecb, Efb, and SCIN block the interaction on a molecular level. Most likely they have different inhibition strategies. Upon binding to the C3d part (TED domain) of C3b, Efb-C induces conformational changes within C3b (Hammel et al. 2007a) which

may result in the impaired recognition by CR1. SCIN binds to the N-terminal tail of C3b α' (Rooijakkers et al. 2009) located in the opposite end than the TED domain. Therefore, it is likely that SCIN inhibits the C3b-CR1 interaction by steric hindrance, which is the suggested mechanism of the inhibition of C3b-CRIg interaction by SCIN (Wiesmann et al. 2006; Jongerius et al. 2010b).

Since CR3 on neutrophils is known to be important for elimination of Gram-positive bacteria (Fällman et al. 1993; Jongstra-Bilen et al. 2003; Nilsson et al. 2005), it is most likely important for *S. aureus* and other Gram-positive bacteria to avoid iC3b depositions, as the findings of the studies III and IV suggest.

On host surfaces C3b depositions are rapidly degraded to C3dg by CR1 expressed on the cell surface or on adjacent erythrocytes. The generated C3dg can bind to CR2 on B cells and in a co-receptor complex (CR2-CD19-CD81) lowers the threshold for activation leading to stimulation of antibody production, which is an important bridge between innate and adaptive immunity (Dempsey et al. 1996). The study IV revealed that Ecb blocked the cofactor activity of CR1 in cleavage of C3b in a similar manner as FH was shown to do in the study III. This is an important finding since it indicates that *S. aureus,* by preventing the formation of C3dg, targets both the innate and adaptive immunity. Previous reports show that *S. aureus* has developed several mechanisms to disable the C3d-CR2 interaction, since Efb, Ecb, and Sbi all occupy areas of C3d that is important for CR2 binding (Burman et al. 2008; Ricklin et al. 2008; van den Elsen & Isenman 2011). So far there are not many examples of microbes using this strategy. The UspA1 and UspA2 proteins of *M. catarrhalis* interact with C3d (Hallström et al. 2011), but it is not known whether the binding hampers the CR2-C3d interaction. Neither of the UspA proteins did induce a conformational change in C3, suggesting that they use a different strategy than the staphylococcal proteins Efb and Ecb. Moreover, the UspAs did inhibit generation of C3a that eventually lead to impaired phagocytosis and inflammatory response, demonstrating that these proteins are important virulence factors of *M*. *catarrhalis*. A recent study showed that the C3d part of iC3b plays a major role in recognition by CR3 (Bajic et al. 2013), suggesting an additional explanation why microbial proteins benefit from binding C3d.

In conclusion, by targeting the C3 molecule, microbes inhibit opsonization, MAC-mediated lysis, phagocytosis, generation of the chemotactic fragments C3a and C5a, and to the bridge to adaptive immunity.

6.3 WHY OVERLAPPING EVASION MECHANISMS? *S. AUREUS* AS AN EXAMPLE

A principal goal of immune evasion by pathogenic microbes is to avoid phagocytosis. This goal can be achieved by producing a thick capsule that requires expression of many proteins involving several genes. Another strategy used by *S. aureus,* is to express small molecules that inhibit many steps in innate immunity, including the complement cascade. The majority of these molecules, such as Ecb and Efb studied in the present work, are expressed by most clinical isolates (Jongerius et al. 2010a). On top of that, many of the molecules have multiple functions as illustrated by the Efb protein. Its N-terminal binds fibrinogen and inhibits platelet functions (Palma et al. 2001), whereas the C-terminal end binds C3b and interacts with complement leading to impaired phagocytosis (Jongerius et al. 2010a; Ko et al. 2013) studies III and IV). Another example is Sbi where its C-terminus of secreted Sbi binds C3 (Burman et al. 2008; Smith et al. 2011) and the N-terminus binds IgG when antibody is attached to the cell wall (Smith et al. 2011). Moreover, both Efb and Sbi recruit plasmin and use it to cleave C3 (Koch et al. 2012). The small 9 kDa Ecb molecule enhances two functions of FH in downregulation of C3b (study III) and blocks interaction with CR1 (study III) and CR2 (Ricklin et al. 2008).

Since all the staphylococcal molecules that target the complement cascade are able to inhibit, but not completely block the different stages, they work in concert and provide a powerful attack against the immune system leading to bacterial survival in the human host.

6.4 EXAMPLES OF EVASION STRATEGIES OF MICROBES IN DIFFERENT BODY PARTS

Host–pathogen interactions are constantly responding to changes in immune status and niche availability. Thus different microbial evasion mechanisms are required for survival in distinct body parts.

Most of the bacteria analyzed in this thesis were isolated from blood, except *B. pertussis*. Infection of blood, however, is frequently preceded by colonization of other tissues of the body and the respiratory tract is one of the most important routes of entry for pathogens. Although the airways are continuously exposed to various types of microbes, the mucosal immunity is eliminating the majority of the intruders. The epithelial cells are covered with a glycoproteins-containing mucus layer that traps the microbes and prevents them from attaching to the host cell surface. With the help of ciliated cells most

of the microbes are efficiently moved of out of the area. In addition, secretory cells are generating a wide range of antimicrobial agents such as sIgA, and serum components, including complement (Persson et al. 1998; Greiff et al. 2003).

Often mixed bacteria cause infections in the respiratory tract and bacteria that are not able to escape the complement system can benefit from co-colonization, as illustrated by unencapsulated strains of *H. influenzae*. Some strains of *M. catarrhalis* release vesicles containing UspA that degrade C3 (Nordström et al. 2004), offering C3-depletion and protection from complement attack for also *H. influenzae* in the nearby area (Tan et al. 2007). Interestingly, the study III showed that a serum sensitive strain of *H. influenzae* was protected against complement-mediated killing in the presence of the secreted staphylococcal C3-binding Ecb protein. This mechanism, however, is most likely due to Ecb bound to surface- deposited C3b leading to inhibition of MAC- mediated lysis.

S. aureus seldom causes infection in the airways but more commonly participates in formation of abscesses or even bacteremia and sepsis. After entering the bloodstream, some bacteria, such as *S. aureus,* can form abscesses by dissemination into tissues (Lowy, *et al.,* 1998). The infection leads to inflammatory responses that attract phagocytes to the site and forms pus, which is a collection of dead and live phagocytes, dead tissue and bacteria. Studies suggest that *S. aureus* protects itself by organizing a "pseudocapsule" composed of fibrin deposits (Cheng et al. 2010), which include several virulence proteins. SpA and coagulases interfere with factors from the coagulation cascade, such as fibrinogen and von Willebrand factor (Friedrich et al. 2003; Bjerketorp et al. 2004). In a mouse model, Ecb and Efb contributed to abscess formation in the heart and kidneys (Jongerius et al. 2012). This was most likely caused by modifying immune responses, since *S. aureus* strains lacking Ecb and Efb caused smaller abscesses and enhanced neutrophil infiltration. This is in line with the study IV that showed that Ecb and Efb prevented interaction with neutrophils. Recently, it was demonstrated that *S. aureus* degrades DNA of the neutrophil extracellular traps resulting in apoptosis of macrophages and increases the possibility of bacterial survival inside the abscess (Thammavongsa et al. 2013).

Occasionally, some strains of *S. pneumoniae* and *H. influenzae* may cause invasive diseases such as sepsis and meningitis either by a commensal strain or another more virulent strain. The yeast *C. albicans* is a commensal on the skin, oral cavity, gastrointestinal and urogenital tracts of healthy individuals. Immunocompromised patients typically have low neutrophils or T helper cell levels, but normal complement

concentrations. In those individuals *C. albicans* can penetrate into deeper tissues, which leads to local or systemic infections. The spirochete bacteria *Borrelia* enters the human body via a tick bite and may cause different diseases, such as cardiac, neurological, or arthritic disorders (Burgdorfer et al. 1982; Nadelman & Wormser 1998). Some species can even persist for long periods of times in mammals, indicating that they efficiently evade the host's immune system.

Taken together, microbes have evolved virulence factors needed for the survival in the human body. Strains that are usually not pathogenic for humans can cause severe disease if they access areas of the body that are less well protected.

6.5 VACCINE DESIGN

Host-pathogen research has one obvious major goal - to develop protective means for hosts against pathogens. Microbial virulence factors or proteins that are involved in the escape from the immune system, could be good vaccine candidates. Therefore, microbial proteins mediating complement evasion are attractive vaccine candidates, such as fHbp of *N. meningitidis,* which is the antigen in an approved vaccine that targets group B meningococci (Madico et al. 2006; Granoff 2013).

B. pertussis should theoretically be possible to eradicate, as the situation was for smallpox in 1978, since humans are the only known reservoir. However, the bacteria are persistent and have a high rate of transmission, as for each infection with *B. pertussis* another fifteen transmission events or secondary infections may occur (Bjornstad & Harvill 2005). We now know that new isolates of *B. pertussis* that express proteins different from those included in the vaccine have evolved (Octavia et al. 2011; Advani et al. 2013). One example is a more virulent *B. pertussis* lineage associated with enhanced pertussis toxin production (Mooi et al. 2009). Recently, a new vaccine administered by the nasal route showed promising results in a phase-I study (Thorstensson et al. 2014). That vaccine includes live bacteria with genetically eliminated dermonecrotic toxin and tracheal cytotoxin, as well as genetically detoxified Ptx. In that manner the vaccine mimic the natural infection without causing the disease (Mielcarek et al. 2006).

The FH ligands of the microbes used in the study II are structurally different and some have been extensively studied. PspC has shown potential as a protective antigen in a mouse model against sepsis, pneumonia, and colonization of lungs (Rosenow et al. 1997). The Tuf -protein is involved in peptide elongation during protein synthesis and is

abundantly present in the cytosol of microbes. A fraction of the protein is identified on the cell surface of several microbes and is therefore included in the group of so-called "moonlighting proteins" (Jeffery 1999; Kunert et al. 2007). Although surface-attached Tuf on *P. aeruginosa* binds FH, its contribution in serum survival is not known. In future experiments a mutant strain lacking Tuf should be analyzed for its sensitivity to human serum before it can be considered as a vaccine candidate.

Pra1 is located on the surface of yeast cells and hyphae of *C. albicans*. In addition, it is found in the culture supernatant. Secreted Pra1 is demonstrated to bind C3 and inhibit the cleavage of C3 into C3b and C3a (Luo et al. 2010), and furthermore, it blocks CR3 on neutrophils (Soloviev et al. 2007). The surface attached Pra1 is shown to bind the soluble complement regulators FH FHL-1, and C4BP (Luo et al. 2009; Luo et al. 2011). Together the secreted and surface-bound Pra1 inhibits C3b deposition, impairs the recruitment of phagocytes and blocks phagocytosis by neutrophils, as well as it inhibits the inflammatory response (Soloviev et al. 2007; Luo et al. 2009; Luo et al. 2010; Luo et al. 2011). Therefore, neutralization of this multifunctional protein would most likely inhibit the survival of *C. albicans*. Vaccines against *C. albicans* and *P. aeruginosa* would be useful as tailored treatments for immunocompromised individuals and patients suffering from cystic fibrosis, respectively.

OspE is proposed to be a good vaccine candidate since it is essential for infection, it is expressed by the majority of clinical isolates and it is highly conserved (Hellwage et al. 2001; Bhattacharjee et al. 2013).

Study II demonstrated that different microbes bound FH in a similar manner and the binding site was mapped on a molecular level. Since membrane proteins often are rather large proteins, this information is essential in future work to design small molecules that could block the interaction site thereby neutralizing several microbes. A challenge is that many of the virulence factors are human-specific, which complicates the analysis since using a valid animal model is not always available.

Often FH binding correlates with bacterial survival in serum, as demonstrated for *S. pyogenes* and *N. meningitidis* (Madico et al. 2006; Haapasalo et al. 2008). Some strains of *S. pyogenes* strongly bind FH5-7. Since FH5-7 lacks a regulatory site, the fragment has been proposed as a therapeutic molecule. By adding FH5-7, FH binding to the microbial protein is blocked (Haapasalo et al. 2012) leading to impaired phagocytosis. Exploiting a human protein minimizes the chances for an antigenic response. This approach would not be possible for FH19-20 since in the studies II and III it was found that FH19-20

enhances the binding of microbial protein to C3b leading to an effect in favor of the microbe.

7 CONCLUSIONS

The innate immunity, including the complement system, is efficient in the first line protection against microbial infections. Complement can directly destroy the microbes by MAC-mediated lysis and recruit and activate phagocytes, such as neutrophils, to the site of infection. In order to survive within humans, microbes have evolved various and distinct strategies to evade the complement attack. For example, several microbes acquire soluble complement regulators. The present studies demonstrated the importance of binding of the C-terminal domain of the main AP regulator FH to bacteria.

By binding to FH19-20, the respiratory tract pathogen *B. pertussis* can escape the AP of complement and increase the survival in human serum. It was shown at a molecular level that a common site on domain 20 of FH was essential for Gram-negative, including *B. pertussis*, and Gram-positive bacteria as well as the yeast *C. albicans* in order to escape from complement attack. Binding of microbial proteins to FH demonstrated an enhanced C3b:FH interaction leading to increased downregulation of C3b that eventually results in increased survival of the microbes.

The important pathogen *S. aureus* does not directly bind FH and these studies gave an explanation on how it uses soluble C3b-binding molecules to recruit host FH. Importantly, the functional consequences of the C3b:FH:Ecb complex were demonstrated both on a protein level and for bacterial survival in serum. Furthermore, the studies demonstrated how *S. aureus* avoids recognition by CR1 and subsequently phagocytosis by neutrophils.

A better understanding of the host-pathogen interaction is essential for the development of new vaccines, novel antimicrobial compounds and new mechanisms to fight against microbial infections. The results in this thesis revealed detailed knowledge on both functional and structural levels of host-pathogen interactions and provide important contribution to this research field.

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